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14. ABSTRACT

Autophagy is a highly conserved and regulated cellular process employed by living cells to degrade proteins and organelles as a response to metabolic stress. We have previously reported that eEF-2 kinase (also known as Ca2+/calmodulin-dependent protein kinase III) can positively modulate autophagy and negatively regulate protein synthesis. The purpose of this project was to determine the role of the eEF-2 kinase-regulated autophagy in the development and progression of breast cancer and the response of breast cancer cells to inhibitors of growth factor signaling. We found that nutrient depletion or growth factor inhibitors activated autophagy in human breast cancer cells, and the increased activity of autophagy was associated with a decrease in cellular ATP and an increase in activities of AMP kinase and eEF-2 kinase. Silencing of eEF-2 kinase relieved the inhibition of protein synthesis, led to a greater reduction of cellular ATP, and blunted autophagic response. We further showed that suppression of eEF-2 kinase-regulated autophagy impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival, and enhanced the efficacy of the growth factor inhibitors such as trastuzumab, gefitinib, and lapatinib. The results of our study provide new evidence that activation of eEF-2 kinase-mediated autophagy plays a protective role for cancer cells under metabolic stress conditions, and that targeting autophagic survival may represent a novel approach to enhancing the effectiveness of growth factor inhibitors.

15. SUBJECT TERMS

elongation factor-2 kinase; autophagy; metabolic stress; growth factor inhibitor; human breast cancer cells

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INTRODUCTION

Autophagy is a highly conserved process by which cytoplasm and organelles are digested via autophagosomes and autolysosomes and cellular components are recycled for energy utilization [1, 2]. During starvation or growth factor deficiency, autophagy may serve as a temporary survival mechanism by providing an alternative energy source. Autophagy can also optimize nutrient utilization in rapidly growing cells when faced with hypoxic or metabolic stresses, thus contributing to cancer cell survival [3-5]. Elongation factor-2 (eEF-2) kinase, a Ca^{2+}/calmodulin-dependent protein kinase, acts as a negative regulator of protein synthesis: this kinase phosphorylates eEF-2, a 100 kDa protein that mediates the translocation step in peptide-chain elongation by inducing the transfer of peptidyl-tRNA from the ribosomal A to P site; phosphorylation of eEF-2 at Thr56 by eEF-2 kinase decreases the affinity of the elongation factor for ribosome and terminates elongation [6]. Our previous studies demonstrated that eEF-2 kinase might be a central component of the mammalian macroautophagy pathway that is activated in response to nutrient deprivation [7, 8]. The role of eEF-2 kinase in the regulation of stress-induced autophagy has further been confirmed by others [9]. Since protein synthesis is a major energy-consuming process, termination of protein synthesis and induction of autophagy via activation of eEF-2 kinase should conserve energy and support cell survival during time of metabolic stress. Moreover, eEF-2 kinase has been found to be overexpressed and its activity increased in multiple breast cancer cell lines and human breast cancer specimens as compared to adjacent normal tissue [10]. The members of the EGFR family such as EGFR/HER1 and HER2/erB2 represent attractive targets for therapeutic intervention in treatment of cancer, due to the roles of these receptor tyrosine kinases in stimulating oncogenic signaling pathways and in the development and progression of cancers [11-13]. Aberrant expression or activity of the EGFR family receptor tyrosine kinases is encountered in many types of malignancies including breast cancers. Indeed, the EGFR tyrosine kinase inhibitors such as lapatinib and gefitinib, and the HER2/neu-targeted agent trastuzumab, have been shown to possess notable antitumor activity in several types of cancers [14]. These drugs can specifically bind to the receptors with high affinity, resulting in blockade of the downstream signaling pathways and inhibition of tumor growth. Nevertheless, refractoriness to these growth factor inhibitors is common [15, 16]. For instance, in patients with HER2-positive metastatic breast cancers, the response rate of trastuzumab is only ~26 % [17]. Thus, understanding of the mechanisms underlying the insensitivity to the growth factors inhibitors and developing approaches to sensitizing tumor cells will make these drugs more valuable in treating patients with breast cancer. The purpose of our study was to reveal the patho-physiological and pharmacological roles, and regulation of autophagy in breast cancer. With the support of this grant, we determined: 1) ***the importance of autophagy in the life and death of breast cancer cells; 2) the role of autophagy in the sensitivity of breast cancer to treatment***. Our study shows that the eEF-2 kinase-mediated autophagy plays a cytoprotective role in breast cancer cells treated with growth factor inhibitors, and inhibiting autophagic survival can modulate sensitivity to these therapeutic agents.

BODY

Task 1 To determine the importance of autophagy in the life and death of breast cancer cells.

In this Task, we have discovered:

1) Nutrient deprivation and growth factor inhibitors activated autophagy in human breast cancer cells

To determine the effect of metabolic stress on autophagy, we first treated the human breast cancer cells MCF-7 with DPBS, and then examined autophagic activity in the treated cells. As shown in Fig. 1A, nutrient deprivation increased the level of LC3-II, a specific marker of autophagic activity. Autophagosome formation was confirmed by GFP-LC3 puncta localization (Fig. 1B). We further found that MCF-7 and MDA-MB-468 breast cancer cells treated with the growth factor inhibitors, gefitinib and lapatinib, showed an increased in LC3-II amount in a dose-dependent manner, as determined by Western blot (Fig. 1C and D). The effects of gefitinib and lapatinib on autophagy were verified using GFP-LC3 cleavage assay (Fig. 1E). These observations suggest that activation of autophagy may represent a cellular response to metabolic stress, including treatment with growth factor inhibitors.

2) Nutrient deprivation caused a reduction of protein synthesis and cellular ATP

To ascertain whether a causal relationship exists between activation of autophagy and metabolic stress in breast cancer cells, we measured protein synthesis activity and ATP level in MCF-7 cells subjected to nutrient starvation. As shown in Fig. 2, treatment of the cells with DPBS (Dulbecco's Phosphate-Buffered Saline) caused a marked decrease in protein synthesis (Fig. 2A) and ATP content (Fig. 2B). Cellular response to energy stress was also evidenced by an increased phosphorylation on Thr172 of AMPK, an intracellular energy sensor that is activated when cellular energy level decreases (Fig. 2C).

3) Nutrient deprivation activated eEF-2 kinase through the mTOR/S6 kinase pathway

We determined the effects of nutrient deprivation on the activity of eEF-2 kinase, a unique calmodulin-dependent enzyme that inhibits protein synthesis and activates autophagy, and on the signaling molecules associated with the regulation of the activity of this kinase. Fig. 3A shows that treatment of MCF-7 cells with DPBS activated eEF-2 kinase, as measured by the phosphorylation of EF-2, the substrate for the kinase. Activation of eEF-2 kinase was also manifested in an increased auto-phosphorylation on Ser398, which is known to positively regulate the activity of this kinase, and in a decreased phosphorylation of the kinase on Ser366, a site known to negatively regulate the activity of this enzyme (Fig. 3A). The activity of S6 kinase, a key translation controller downstream of mTOR, was decreased, as evidenced by a decrease in the phosphorylation on Thr389 of S6 kinase (Fig. 3B). The activity of 4EBP1, a translation repressor, was increased, as shown by a decrease in the phosphorylation of this protein (Fig. 3B).

4) eEF-2 kinase was involved in autophagy induction and ATP reduction in response to nutrient depletion

To analyze whether eEF-2 kinase plays a regulatory role in nutrient starvation-induced reduction of protein synthesis and ATP content and in activation of autophagy in breast cancer cells stressed with nutrient depletion, we silenced EF-2 kinase expression using siRNA, and then measured ATP level, protein synthesis and autophagy activity following treatment with DPBS. As shown in Fig. 4A, inhibition of eEF-2 kinase by siRNA decreased starvation-induced autophagy. Inhibition of eEF-2 kinase also resulted in mitigation of the nutrient depletion-induced inhibition of protein synthesis (Fig. 4B) and led to further reduction of ATP levels (Fig. 4C). These results further support a role for eEF-2 kinase in activating autophagy in metabolically stressed tumor cells.

5) Suppression of autophagy decreased growth and survival of metabolically stressed breast cancer cells

To further test our hypothesis that autophagy plays a pro-survival role in response to a compromised supply of cellular nutrients and growth factors during breast cancer development and progression, we knocked down eEF-2 kinase, beclin-1 or ATG5 (two of the key autophagy-related genes) in MCF-7 cells (Fig. 5A), and then compared the growth and survival of these autophagy-deficient cells with that of the cells transfected with a non-targeting RNA in serum-free medium or HBSS. As shown in Fig. 5B, suppression of autophagy by knockdown of those autophagy-regulated genes hindered the tumor cell growth in the absence of serum. Knockdown of eEF-2 kinase, beclin-1 or ATG5 also caused more death of MCF-7 cells cultured in HBSS (Fig. 5C). The autophagy inhibitor, 3-MA, was used as a control and showed a similar inhibitory effect on cell growth and survival in the absence of serum or nutrients (Fig. 5B and C).

However, in this Task, we did not observe the effects of autophagy on anchorage independent growth and invasion of breast cancer cells, as proposed in "b" section of this task. Effects of autophagy on metastasis of breast cancer in animals were not observed either. This is likely due to the complexities of the processes of both autophagy and tumor metastasis, as was also reported by others [18].

Task 2 To determine the role of autophagy in the sensitivity of breast cancer to treatment.

In this Task, we have discovered:

Inhibition of eEF-2 kinase sensitized breast cancer cells to growth factor inhibitors

To determine whether suppression of the eEF-2 kinase-mediated autophagy alters sensitivity of tumor cells to growth factor inhibitors that are in clinical use, we first transfected MCF-7 cells with an eEF-2 kinase-targeted siRNA or a non-targeting RNA, and then treated the transfected cells with a series of concentrations of gefitinib or lapatinib. Fig.6 A and B show that silencing of EF-2 kinase expression increased sensitivity of MCF-7 cells to gefitinib and lapatinib. Similar results were observed with the human breast cancer cells MDA-MB-468 (Fig. 6C and D). Inhibition of autophagy by an eEF-2 kinase-targeted siRNA also enhanced the cytotoxic effects of a small molecule EGFR/ErbB-2 inhibitor (EEi) and trastuzumab, an anti-Her2 therapeutic antibody (Table

1). Combined use of the inhibitors of growth factor and autophagy produced combination indexes (CIs) smaller than 1 (Table 1), indicating a synergism between the actions of those treatments. Study of the effects of autophagy on the efficacy of tamoxifen, OH-tamoxifen, and ICF 164 384 in breast cancer cells are in progress.

KEY RESEARCH ACCOMPLISHMENTS

- We found that growth factor inhibitors activated autophagy in human breast cancer cells.
- We showed nutrient deprivation caused a reduction of protein synthesis and cellular ATP.
- We showed that nutrient deprivation activated eEF-2 kinase through the mTOR/S6 kinase pathway.
- We demonstrated that eEF-2 kinase was involved in autophagy induction and ATP reduction in response to nutrient depletion.
- We observed that suppression of autophagy decreased growth and survival of metabolically stressed breast cancer cells.
- We found that inhibition of eEF-2 kinase sensitized breast cancer cells to growth factor inhibitors.
- We found that *beclin 1* is a putative target for miR-30a.
- We showed that miR-30a negatively regulates *beclin 1* expression.
- We demonstrated the effect and mechanism of miR-30a on autophagic activity.

REPORTABLE OUTCOMES

Manuscript

Zhu H, Wu H, Liu XP, Li B, Chen Y, Ren XC, Liu CG, Yang JM: Regulation of Autophagy by a *Beclin 1*-targeted MicroRNA, miR-30a, in Cancer Cells. Autophagy 5(6): 816-823, 2009

Cheng Y, Li H, Ren XC, Niu TK, Hait WN and Yang JM: Cytoprotective Effect of the Elongation Factor-2 Kinase-mediated Autophagy in Breast Cancer Cells Subjected to Growth Factor Inhibition. PLoS ONE 5(3): e9715, 2010

Abstracts

Li HM, Yang JM, Jin SK, Hait WN: Targeting Autophagic Survival by Inhibiting Elongation Factor-2 Kinase in Human Breast Cancer Cells. Proc Amer Assoc Cancer Res 48: 4917, 2007.

Li HM, Hait WN, Yang JM: Targeting autophagic survival pathway sensitizes human breast cancer cells to growth factor antagonists. Proc Amer Assoc Cancer Res 49: 4935, 2008.

Zhu H, Wu H, Liu X, Chen Y, Ren XC, Liu CG, Yang JM: Regulation of Autophagy by the *Beclin 1*-targeted MicroRNA in Cancer Cells. Proc Amer Assoc Cancer Res 50: 387, 2009.

Degree obtained that are supported by this award

None

Employment or research opportunities

Mike H Li, Ph.D., post-doctoral trainee (May, 2006 – February, 2008)

Ting-Kuang Niu, Ph.D., post-doctoral trainee (August, 2008 – December, 2009)

Yan Cheng, Ph.D., post-doctoral trainee (June, 2009 –)

CONCLUSIONS

The results of our study provide new evidence that activation of eEF-2 kinase-mediated autophagy plays a protective role for cancer cells under metabolic stress conditions, and that targeting autophagic survival may represent a novel approach to enhancing the effectiveness of growth factor inhibitors.

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Supporting Data

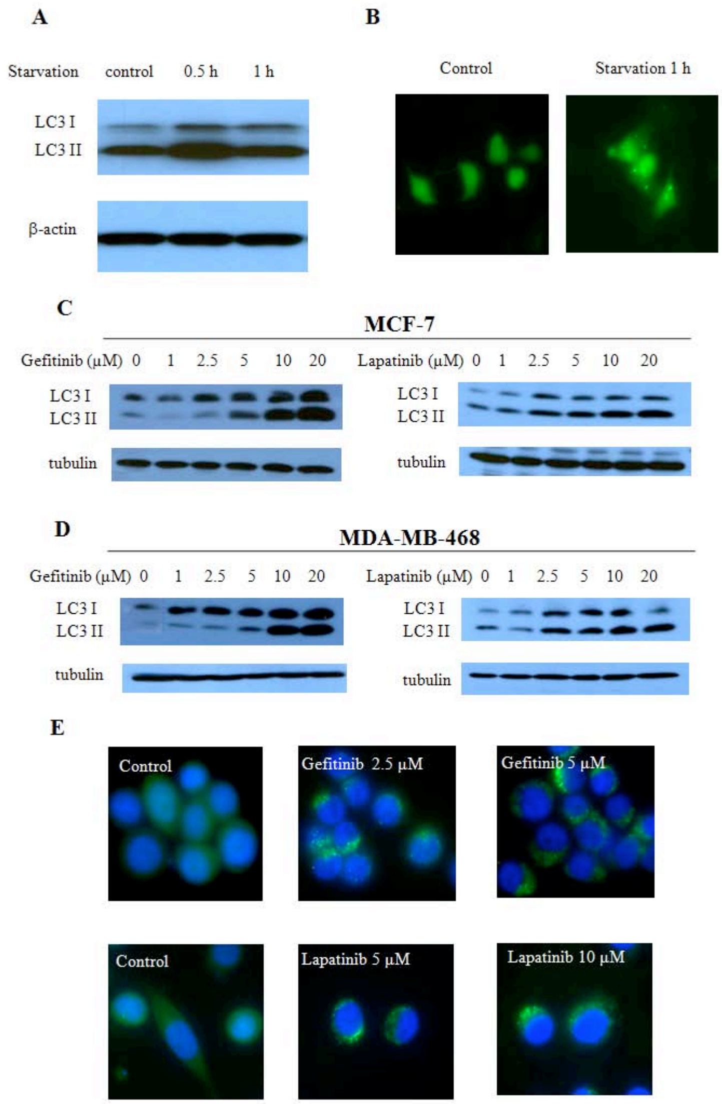

Figure 1. Effect of nutrient deprivation and growth factor inhibitors on autophagy in human breast cancer cells. (**A**) MCF-7 cells were treated with DPBS for the indicated times. At the end of treatment, formation of the autophagy marker LC3-II was detected by immunoblotting with an anti-MAP-LC3 antibody. (**B**) MCF-7 cells transfected with 3μg of GFP-LC3 plasmid were treated or untreated with DPBS for 1h, and then observed under a fluorescent microscope. A representation of GFP-LC3 positive cells was shown. (**C, D**) MCF-7 (C) or MDA-MB-468 (D) cells cultured in medium containing 10% FBS were treated with 0.5, 1, 2.5, 5 or 10μM gefitinib or lapatinib for 24h, and the LC3-II level was examined by immunoblotting. (**E**) MDA-MB-468 cells were transfected with a GFP-LC3-expressing vector, and then treated with the indicated concentration of gefitinib or lapatinib in the presence of the lysosomal protease inhibitors E64d (10 μg/ml) and pepstain A (10 μg/ml). At the end of treatments, cells were fixed with 4% formaldehyde for 15 min. To determine the autophagic response, cells were inspected at 60x magnification for numbers of GFP-LC3 puncta.

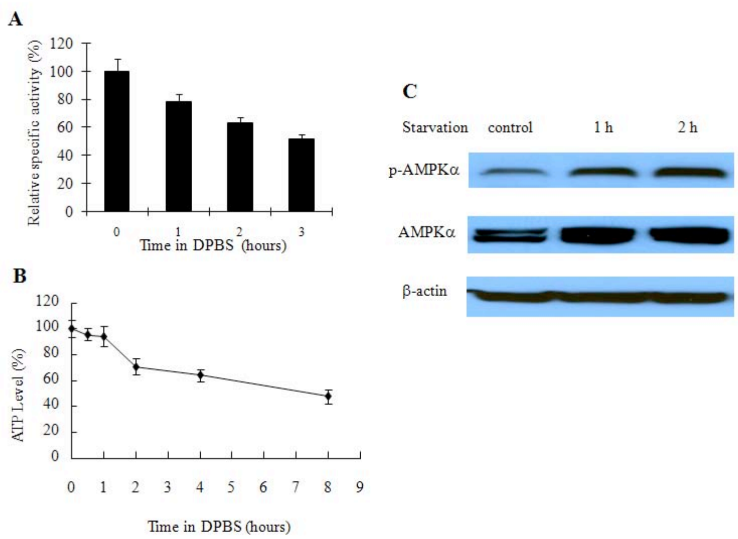

Figure 2. Effect of nutrient deprivation on protein synthesis and cellular ATP in MCF-7 cells. (**A**) MCF-7 cells were treated with DPBS for the indicated times and the rate of protein synthesis was measured by labeling the cells with 25 μCi/ml of EasyTag EXPRESS [^{35}S] protein labeling mix and liquid scintillation counting, as described in "Material and Methods". The specific activity of protein synthesis was determined by the amount of incorporated ^{35}S-methionine/cysteine per mg of total protein per min, and relative activities at indicated times of starvation were calculated as percent of control. (**B**) MCF-7 cells were treated with DPBS for the indicated times and ATP content was measured using the ATPlite™ Luminescence Assay Kit. (**C**) AMPK activity was determined by Western blot analysis of phospho-AMPK using an anti-phospho-AMPK antibody, as described in "Material and Methods". Actin was used as a loading control. Results shown are the representative of three similar experiments; each bar or point represents mean ± SD of quadruplicate determinations.

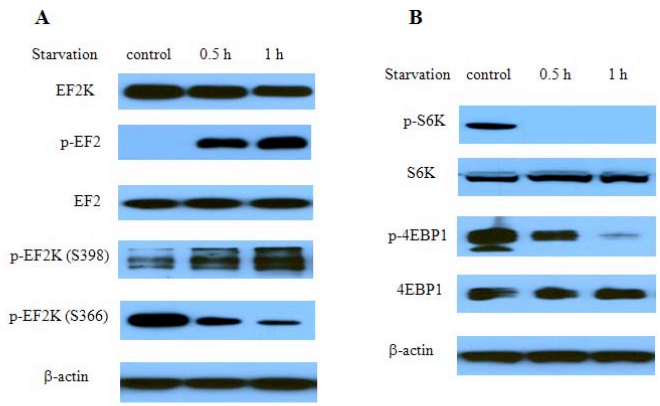

Figure 3. Effect of nutrient deprivation on eEF-2 kinase activity and the associated signaling molecules. (**A**) MCF-7 cells were treated with DPBS for the indicated times, and the levels of eEF-2 kinase, p-EF2, EF2, p-eEF2 kinase (S398) and p-eEF2 kinase (S366) were examined by Western blot using the respective antibodies. (**B**) MCF-7 cells were treated with DPBS for the indicated times, and p-S6 kinase, S6 kinase, p-4EBP1, and 4EBP1 were examined by Western blot using the respective antibodies. β-actin was used as a loading control. Results shown are the representative of three similar experiments.

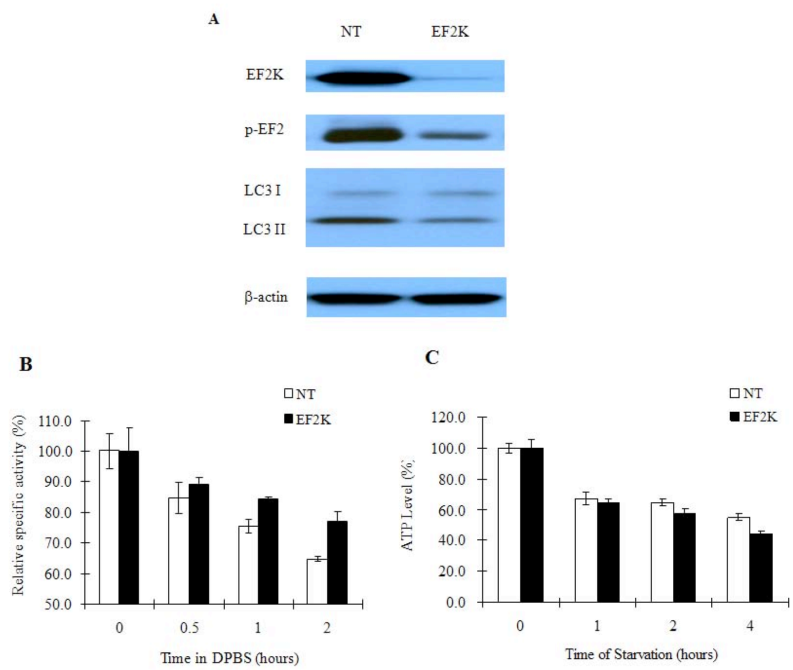

Figure 4. Inhibition of eEF-2 kinase blunts autophagy, mitigates inhibition of protein synthesis and hastens reduction of cellular ATP. (**A**) MCF-7 cells were transfected with a non-targeting RNA or an eEF-2 kinase-targeted siRNA (100 nM) for 72 h, and then treated with DPBS for 1 h. eEF-2 kinase, phosphor-EF-2, and the autophagy marker, LC3-II, were detected by Western blot. (**B**) MCF-7 cells with or without silencing of eEF-2 kinase were treated with DPBS; at the indicated times, cells were harvested for protein synthesis assay. (**C**) MCF-7 cells transfected with 50 nM of NT RNA or an eEF-2 kinase siRNA were seeded in 96-well tissue culture plates (1 x10^4 cells per well). Forty-eight h later, the cells were starved in DPBS for 1 h, 2 h and 4 h. Cells were collected at the end of starvation for ATP assay. Results shown are the representative of three similar experiments; bars represent mean ± SD of quadruplicate determinations.

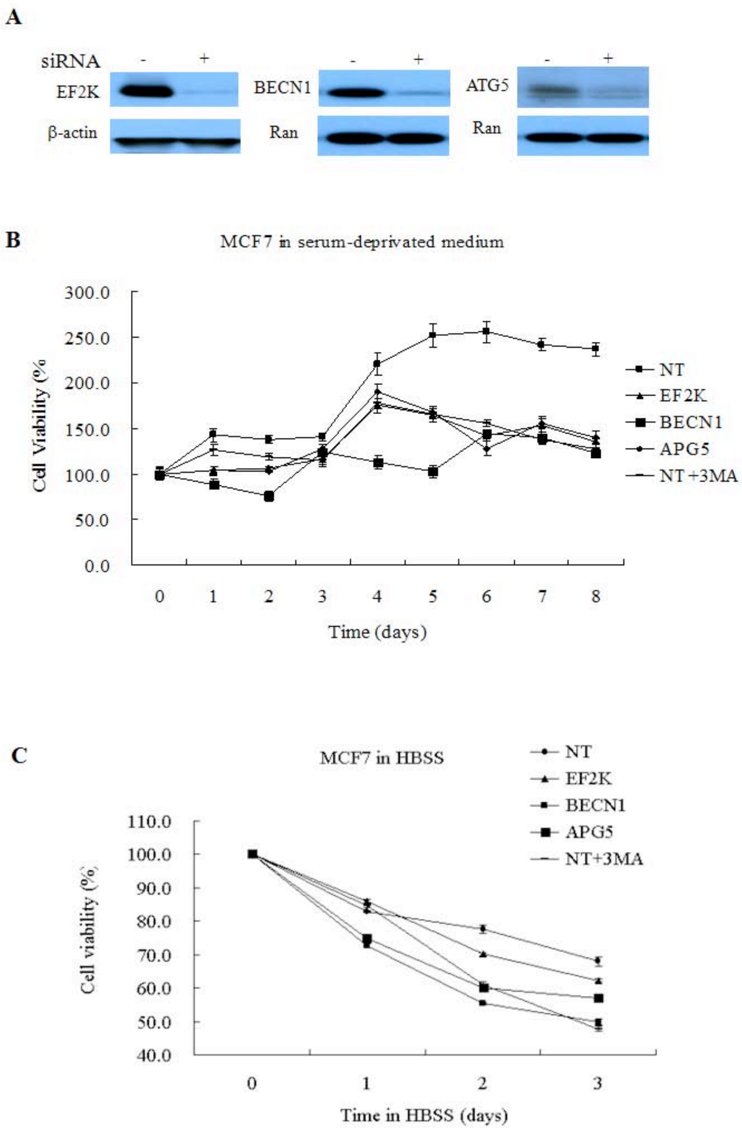

Figure 5. Effects of autophagy suppression on growth and survival of human breast cancer cells. **(A)** MCF-7 cells were transfected with a non-targeting RNA or a siRNA targeting eEF-2 kinase, beclin1, or ATG5. Expressions of eEF-2 kinase, beclin1, or ATG5 were determined by Western blot using the respective antibodies. β-actin or Ran was used as a loading control. (**B**) MCF-7 cells treated with 3-MA or with siRNA targeting eEF-2 kinase, beclin1, ATG5 or a non-targeting RNA were seeded with 10% FBS RPMI 1640 medium in 96-well culture plates (3 x10^3 cells per well). After overnight incubation, medium was changed to serum-free medium. Cell viability was determined at the indicated times using MTT assay. (**C**) MCF-7 cells treated with 3-MA or with siRNA targeting eEF-2 kinase, beclin1, ATG5 or a non-targeting RNA were seeded with 10% FBS RPMI 1640 medium in 96-well culture plates (3 x10^3 cells per well). After overnight incubation, medium was changed to HBSS. Cell viability was determined at the indicated times using MTT assay. Results shown are the representative of three similar experiments; each point represents mean ± SD of quadruplicate determinations.

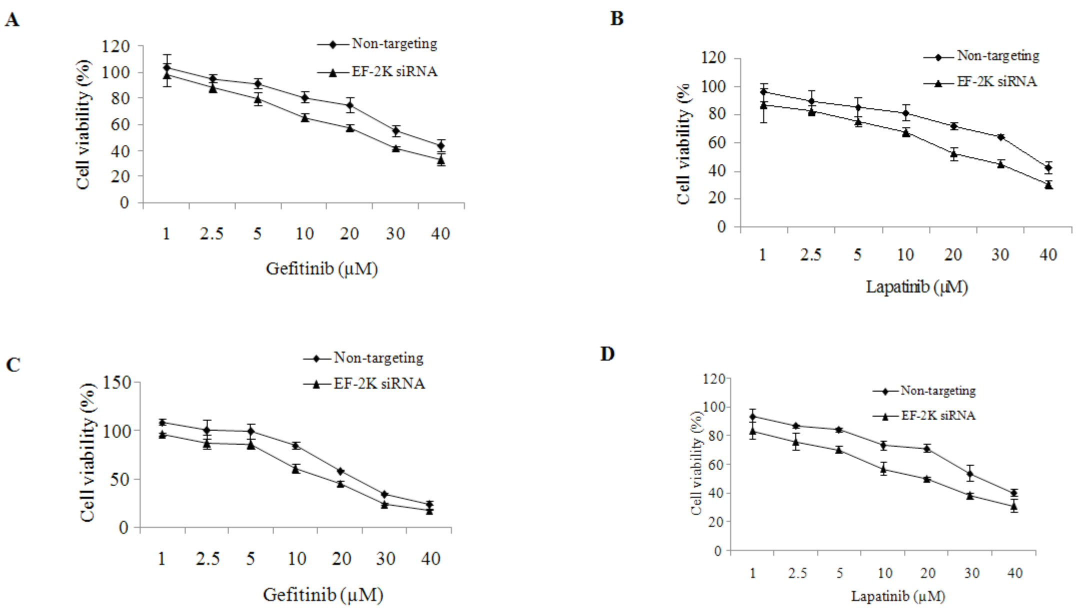

Figure 6. Effects of eEF-2 kinase silencing on sensitivity of human breast cancer cells to growth factor inhibitors. MCF-7 (**A, B**) and MDA-MB-468 (**C, D**) cells transfected with an siRNA targeting eEF-2 kinase or a non-targeting RNA were cultured in RPMI 1640 or DMEM media supplemented with 10% fetal bovine serum at 37 ^{o}C in a humidified incubator (5% CO_2 and 95% air), and then treated with a series of concentrations of gefitinib (**A, C**) or lapatinib (**B, D**) for 48h. At the end of treatment, cell viability was determined using MTT assay. Results shown are the representative of three similar experiments; each point represents mean ± SD of quadruplicate determinations.

Table 1. CompuSyn analysis of the combinations of growth factor inhibitors and eEF-2 kinase siRNA

Cell line	Combination	CI values
MCF7	Gefitinib : eEF-2K siRNA	0.45
	Lapatinib : eEF-2K siRNA	0.42
	EEi : eEF-2K siRNA	0.51
	Trastuzumab: eEF-2K siRNA	0.60
MDA-MB-468	Gefitinib : eEF-2K siRNA	0.40
	Lapatinib : eEF-2K siRNA	0.53
	EEi : eEF-2K siRNA	0.65
	Trastuzumab: eEF-2K siRNA	0.67

Combination index (CI) was calculated using the computer program, CompuSyn.

Appendices

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Research Paper

Regulation of autophagy by a *beclin 1*-targeted microRNA, miR-30a, in cancer cells

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†These authors contributed equally to this work.

Abbreviations: miRNA, microRNA; UTR, untranslated region; HBSS, hank's buffered salt solution; LC3, microtubule-associated protein 1 light chain 3; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, tris-buffered saline

Key words: *beclin 1*, autophagy, microRNA, miR-30a, gene expression

***beclin 1*, the mammalian homologue of the yeast *Atg6*, is a key autophagy-promoting gene that plays a critical role in the regulation of cell death and survival of various types of cells. However, recent studies have observed that the expression of *beclin 1* is altered in certain diseases including cancers. The causes underlying the aberrant expression of *beclin 1* remain largely unknown. We report here that microRNAs (miRNAs), a class of endogenous, 22–24 nucleotide noncoding RNA molecules able to affect stability and translation of mRNA, may represent a previously unrecognized mechanism for regulating *beclin 1* expression and autophagy. We demonstrated that *beclin 1* is a potential target for miRNA miR-30a, and this miRNA could negatively regulate *beclin 1* expression resulting in decreased autophagic activity. Treatment of tumor cells with the miR-30a mimic decreased, and with the antagomir increased, the expression of *beclin 1* mRNA and protein. Dual luciferase reporter assay confirmed that the miR-30a binding sequences in the 3'-UTR of *beclin 1* contribute to the modulation of *beclin 1* expression by miR-30a. Furthermore, inhibition of *beclin 1* expression by the miR-30a mimic blunted activation of autophagy induced by rapamycin. Our study of the role of miR-30a in regulating *beclin 1* expression and autophagy reveals a novel function for miRNA in a critical cellular event with significant impacts in cancer development, progression and treatment, and in other diseases.**

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Introduction

Autophagy, a conserved, programmed response to metabolic and environmental stress found in yeast, plants, worms, flies, mice and man,[1] has been known to play a critical role in the regulation of survival and death of various types of cells.[2] Recent studies have implicated autophagy in a number of physiologic and pathophysiologic processes such as aging, cancers and neurodegenerative diseases.[3] The process of autophagy involves formation of double-membrane vesicles (autophagosomes) that engulf organelles and cytoplasm, then fuse with the lysosome to form the autolysosme, where the contents are degraded and recycled for protein and ATP synthesis.[4,5] The formation of the autophagosome is mediated by a series of autophagy-promoting gene products that function at different stages of autophagy.[6] *beclin 1*, the mammalian homologue of the yeast *Atg6*, is a key autophagy-promoting gene whose product is part of a lipid kinase (class III Phosphoinositide 3-kinase) complex that participates in the early stage of autophagosome formation.[7,8] Beclin 1 is a ~60-kDa coiled-coli protein also able to interact with bcl-2, an anti-apoptotic protein. Although ubiquitously expressed, it has been known that the expression of *beclin 1* is altered in certain diseases. For example, in early Alzheimer disease *beclin 1* expression is decreased;[9] in contrast, neurodegeneration causes upregulation of *beclin 1*.[10] In several types of human cancers, the expressions of *beclin 1*, both protein and mRNA, were also found to be aberrant.[11-13] Yet, the causes underlying the altered expression of this key autophagy-promoting gene remain largely unknown. In the present study we sought to explore the role of microRNAs (miRNAs) in the regulation of expression of *beclin 1*. MiRNAs are a class of endogenous, 22–24 nucleotide RNA molecules with the ability to induce mRNA degradation, translational repression, or both, via pairing with partially complementary sites in the 3' UTR of the targeted genes.[14-17] It is estimated that more than 1,000 miRNAs exist in mammalian cells, and that 30% of all genes are regulated by miRNAs.[18-20] Because of their capacity to target numerous mRNAs, miRNAs can regulate the expression of genes in a number of pathways that are associated with tumor initiation, development and progression.[21,23] We report here that the autophagy-promoting gene *beclin 1* is a potential target for miR-30a, and this miRNA may play a regulatory role in autophagic response through modulating the expression of *beclin 1*.

Results

***beclin 1* is a putative target for miR-30a.** To explore the possible role of miRNAs in regulating autophagy, we first analyzed the miRNA expression profiling in tumor cells treated with HBSS (nutrient deprivation) or rapamycin. Nutrient deprivation and rapamycin treatment are known to activate autophagy in various types of cells.[26,27] Using a miRNA microarray (OSUCCC-microRNA version 4.0) analysis, we observed a differential expression of 13 miRNAs in T98G and MDA-MB-468 cells treated with HBSS or rapamycin, as compared with the untreated cells or the cells treated with vehicle. The heat map of these miRNA expression profiles of those samples is shown as Supplemental data (Fig. S1). We then conducted an in silico search for miRNA binding sites using the PicTar algorithm (http://pictar.bio.nyu.edu). Among those 13 miRNAs that were differentially expressed in the HBSS or rapamycin-treated cells, we found in the 3'-UTR of *beclin 1* the consensus sequences for miR-30a, implying that *beclin 1* is a potential target for miR-30a. To verify the change of miR-30a expression following HBSS or rapamycin treatment, we performed qRT-PCR analysis of the endogenous miR-30a expression. Figure 1 shows a 10% and 35% reduction of miR-30a expression in cells subjected to nutrient depletion or rapamycin treatment, respectively. These results suggest a possible role for this miRNA in targeting *beclin 1* in response to stresses.

MiR-30a negatively regulates *beclin 1* expression. To obtain experimental evidence supporting *beclin 1* gene as a target for miR-30a, we next examined the effect of miR-30a on *beclin 1* expression using a mimic and an antagomir of this miRNA. In these experiments, T98G, MDA-MB-468 and H1299 cells were transfected with a miR-30a mimic or an antagomir, and then the expressions of *beclin 1* mRNA and protein were analyzed by qTR-PCR and western blot, respectively. As shown in Figure 2, transfection with the miR-30a mimic caused a 25–40% decrease in *beclin 1* mRNA (Fig. 2A), and a 25–60% decrease in beclin 1 protein (Fig. 2B); by contrast, treatment with the miR-30a antagomir resulted in a 55–85% increase in *beclin 1* mRNA (Fig. 2A) and a 10–25% increase in beclin 1 protein (Fig. 2B). Using the mimic and antagomir of miR-30a, these experiments demonstrated a suppressive role for this miRNA in *beclin 1* expression.

To validate the predicted consensus sequences for miR-30a in the *beclin 1*-3' UTR, and determine whether these miR-30a binding sequences directly contributed to the negative regulation of *beclin 1* expression, we tested the effects of miR-30a on activity of a reporter gene using the vectors that either contained wild-type miR-30a targeting sequences (psiCHECKTM2-WT-*BECN*-3'-UTR) or deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR) (Fig. 3B).

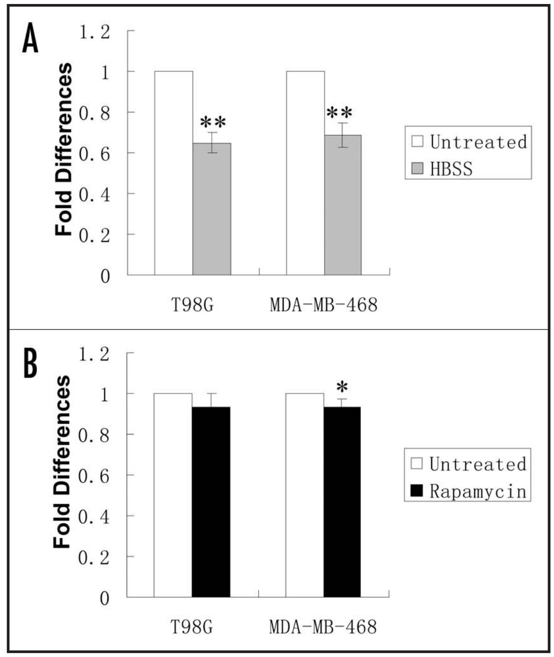

Figure 1. Effects of HBSS and rapamycin on endogenous miR-30a expression. T98G and MDA-MB-468 cells were treated with HBSS for 4 h or rapamycin (200 nM) for 12 h. At the end of treatment, endogenous miR-30a expression was analyzed by Real-time RT-PCR, as described in "Material and Methods." Small nuclear RNA (RNU66) was used as internal control. The expression level of miR-30a was calculated using the MxPro software (Version 4.00, Stratagene). Results shown are the mean ± SD of triplicate determinations from one of three identical experiments. *p < 0.05 vs. control; **p < 0.01 vs. control.

As shown in Figure 3C, cotransfection of T98G, MDA-MB-468 and H1299 cells with 50 nM or 100 nM of the miR-30a mimic resulted in a 40–80% reduction in the activity of the reporter gene vector containing the wild-type miR-30a targeting sequences (psiCHECKTM2-WT-*BECN*-3'-UTR), in comparison to that of the vector with the deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR). In contrast, the nontargeting control RNA did not have any effect on the reporter activity of either of the vectors (Fig. 3C). These results demonstrated that the miR-30a binding sequences in the *beclin 1*-3' UTR is the region required for the miR-30a-mediated inhibition of *beclin 1* expression.

Effect of miR-30a on autophagic activity. Beclin 1 plays an essential role in activating autophagy. To determine the functional consequence of modulation of *beclin 1* expression by miR-30a, we tested the effect of the miR-30a mimic on autophagic response to rapamycin. Figure 4 shows that treatment of T98G cells with rapamycin activated autophagy, as evidenced by the increases in LC3-II amount (Fig. 4A), in double membrane vacuoles in the cytoplasm (Fig. 4B), and in formation of GFP-LC3 aggregation (Fig. 4C). Notably, tumor cells transfected with the mimic of miR-30a showed a remarkable reduction in *beclin 1* expression, and a blunted autophagic response to rapamycin, as evidenced by the lower LC3-II levels (Fig. 4A), fewer double membrane vacuoles (Fig. 4B), and less GFP-LC3 aggregations (Fig. 4C), indicating that inhibition of *beclin 1* expression by miR-30a leads

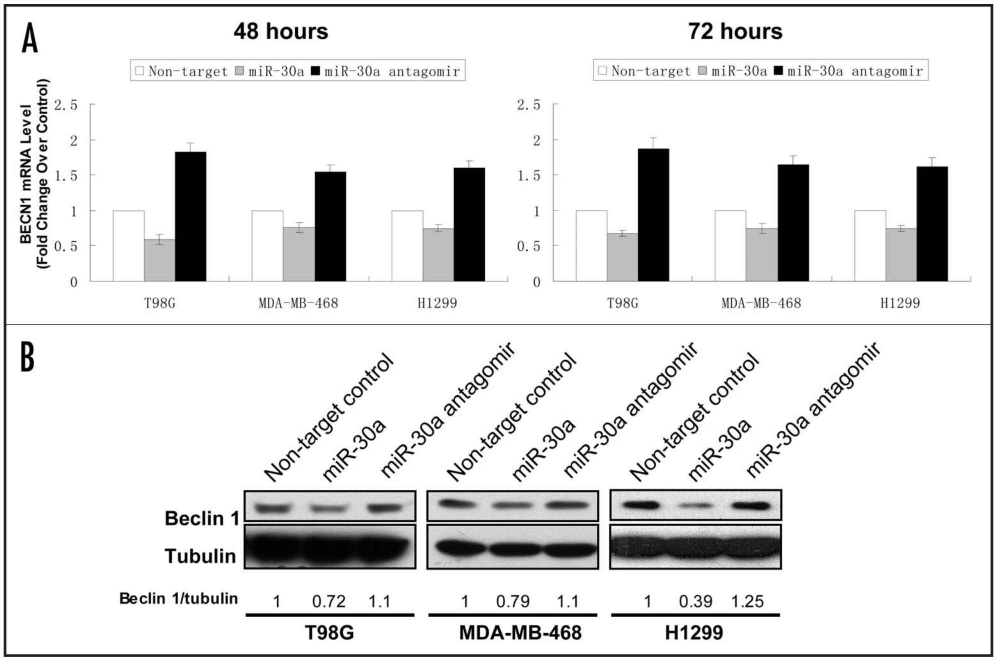

Figure 2. Effect of miR-30a on the expression of *beclin 1*. (A) T98G, MDA-MB-468 and H1299 cells were transfected with a mimic or antagomir of miR-30a (100 nM) or a control RNA (100 nM). Forty-eight and 72 hours later, total RNAs were extracted from the treated cells and quantitative real-time RT-PCR analyses of *beclin 1* mRNA were performed as described in "Material and Methods." *beclin 1* mRNA levels of the cells treated with a control RNA were arbitrarily set at 1, and *beclin 1* mRNA levels of the cells treated with the miR-30a mimic were normalized to the control. Results shown are the mean ± SD of triplicate determinations from one of three identical experiments. (B) T98G, MDA-MB-468 and H1299 cells were treated as described in (A). Forty-eight hours later, cell lysates were prepared from the transfected cells. Equal amounts (25 μg proteins) of cell lysates were separated by 8% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with a monoclonal anti-*beclin 1* antibody. Detection of *beclin 1* was performed using enzyme-linked chemiluminescence. α-tubulin was used as a loading control. Protein expression was quantified using the ImageJ software. Beclin 1/tubulin ratios of the samples treated with a control RNA was arbitrarily set at 1, and the Beclin 1/tubulin ratios of the miR-30a mimic or antagomir-treated samples were normalized to the control. Results shown are the mean ± SD of triplicate determinations from one of three identical experiments.

to suppression of autophagic activity. To confirm that the effect of miR-30a on autophagy was mediated through *beclin 1*, we tested the effect of miR-30a on the resveratrol-induced autophagy in MCF-7 cells, which was reported to be *beclin 1*-independent.[28] Figure 5 shows that miR-30a mimic had no effect on autophagy induced by resveratrol in MCF-7 cells.

Discussion

It is known that altered autophagic activity is associated with a number of diseases such as cancer,[18,29,30] and that the *beclin 1*-mediated autophagy plays an important role in the regulation of cell survival and death.[8,31] However, *beclin 1* expression has been found to be aberrant in certain diseases including cancers.[9-13] How autophagy and *beclin 1* expression are regulated is not fully understood. The current study reports our finding that miRNAs can control the expression of *beclin 1* expression, thereby modulating autophagic activity. Through the use of mimic and antagomir, we demonstrate that the miRNA miR-30a is able to modulate autophagy through inhibiting *beclin 1* expression, and this effect is mediated via the miR-30a consensus sequences contained in the 3'-UTR of *beclin 1*. To our knowledge, this is the first report on the role of miRNAs in regulating autophagy, an important cellular process that is involved in many physiological and pathophysiologic events including cancer.

The role of miR-30a in the regulation of *beclin 1* expression is evidenced by our experiments showing that transfection of tumor cells with the miR-30a mimic resulted in decreases in both mRNA and protein of *beclin 1* (Fig. 2), and in reductions of the activity of a reporter gene plasmid containing the consensus sequences for miR-30a (Fig. 3). By contrast, in the absence of the miR-30a consensus sequences the inhibitory effect of miR-30a on the reporter activity was abolished (Fig. 3C). Furthermore, we showed that transfection of cells with an antagomir of miR-30a caused an increase in *beclin 1* mRNA and protein (Fig. 2), providing additional evidence for a possible role of miR-30a in controlling *beclin 1* expression. Inhibition of *beclin 1* expression by miR-30a leads to suppression of autophagic activity, as transfection with the miR-30a mimic also blunted autophagic response of tumor cells to rapamycin (Fig. 4), an activator of autophagy.[32] Additionally, the role of miR-30a in modulating autophagy and *beclin 1* expression is supported by our analysis of the endogenous miRNA expression, which showed a various degrees of reduction of miR-30a expression in the cells subjected to different treatments (Fig. 1), although Beclin 1 protein levels appeared unchanged following those treatments (Fig. 4A). This is probably because although downregulation of miR-30a can slow down the degradation of *beclin 1* mRNA or/and de-repress translation of Beclin 1 protein, the change of the endogenous miR-30a caused by rapamycin is not sufficient to upregulate Beclin 1 protein level. In fact, an increase in Beclin 1 has not so far been found to be a requisite for activation of autophagy. The results of the current study mainly depend on use of the mimic and antagomir to miR-30a; nevertheless, whether and how endogenous miR-30a actually regulates *beclin 1* expression, and whether the expression of Beclin 1 protein is indeed regulated by miR-30a, would need further investigation. Additionally, the specific effect of miR-30a on Beclin 1-mediated autophagy was supported by our results showing that the resveratrol-induced autophagy in MCF-7 cells, which was reported to be Beclin 1-independent,[28] was not affected by treatment with miR-30a mimic (Fig. 5).

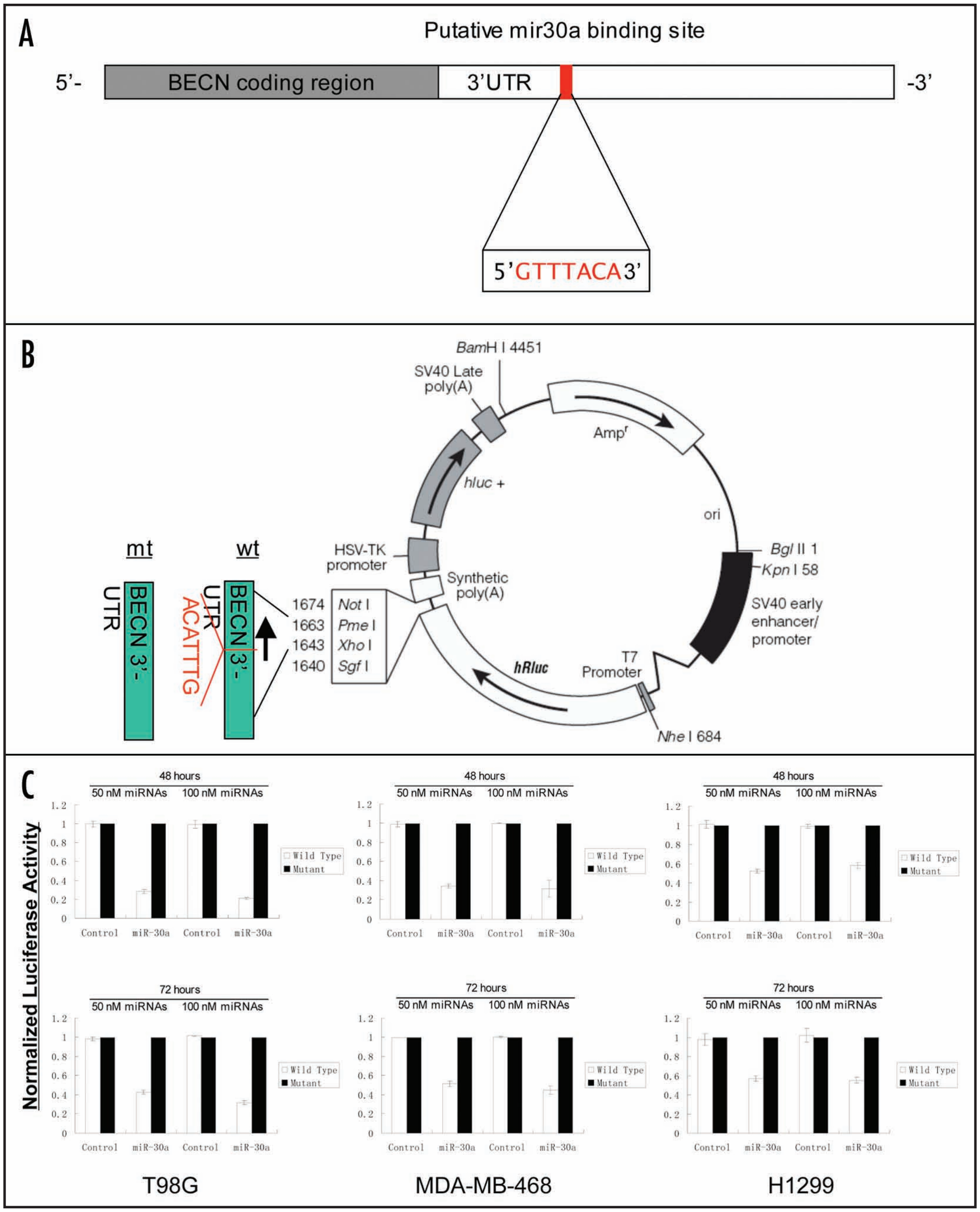

Figure 3. *beclin 1* is a target for miR-30a. (A) The miR-30a consensus sequences in the *beclin 1* 3'-UTR. (B) Construction of the psiCHECKTM2-WT-*BECN*-3'-UTR and psiCHECKTM2-MT-*BECN*-3'-UTR. The 1,574 bp fragment of the *beclin 1* 3'-UTR containing the miR-30a consensus sequences was inserted into the psiCHECKTM2 dual luciferase reporter plasmid at the 3' end of the coding sequence of *R. reniformis* luciferase. The deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR) was generated using mutagenesis PCR method. (C) Luciferase reporter assays. Cells were cotransfected with either psiCHECKTM2-WT-*BECN*-3'-UTR or psiCHECKTM2-MT-*BECN*-3'-UTR vector and a miR-30a mimic or a nontargeting control RNA. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega), and Renilla luciferase activity was normalized to firefly luciferase activity. Results shown are the mean ± SD of triplicate determinations from one of three identical experiments.

In this study we have found that *beclin 1* expression is negatively regulated by miR-30a. As a series of gene products such as *Atg5*, *Atg7* and *Atg10* participate in activating autophagy,[6] it is likely that other autophagy-promoting genes are also regulated by miRNAs, given the pervasive regulatory functions of these small RNA molecules in biology. The results reported here were obtained with cancer cell lines in this study. Nevertheless, aberrant expression of miR-30a and some other miRNAs have been indeed found in human cancers.[33] It would be important and interesting to further analyze if a direct correlation between the aberrant expression of endogenous miR-30a and altered expression of *beclin 1* exists in cancer specimens and cell lines, although such a study would require a statistical analysis of an

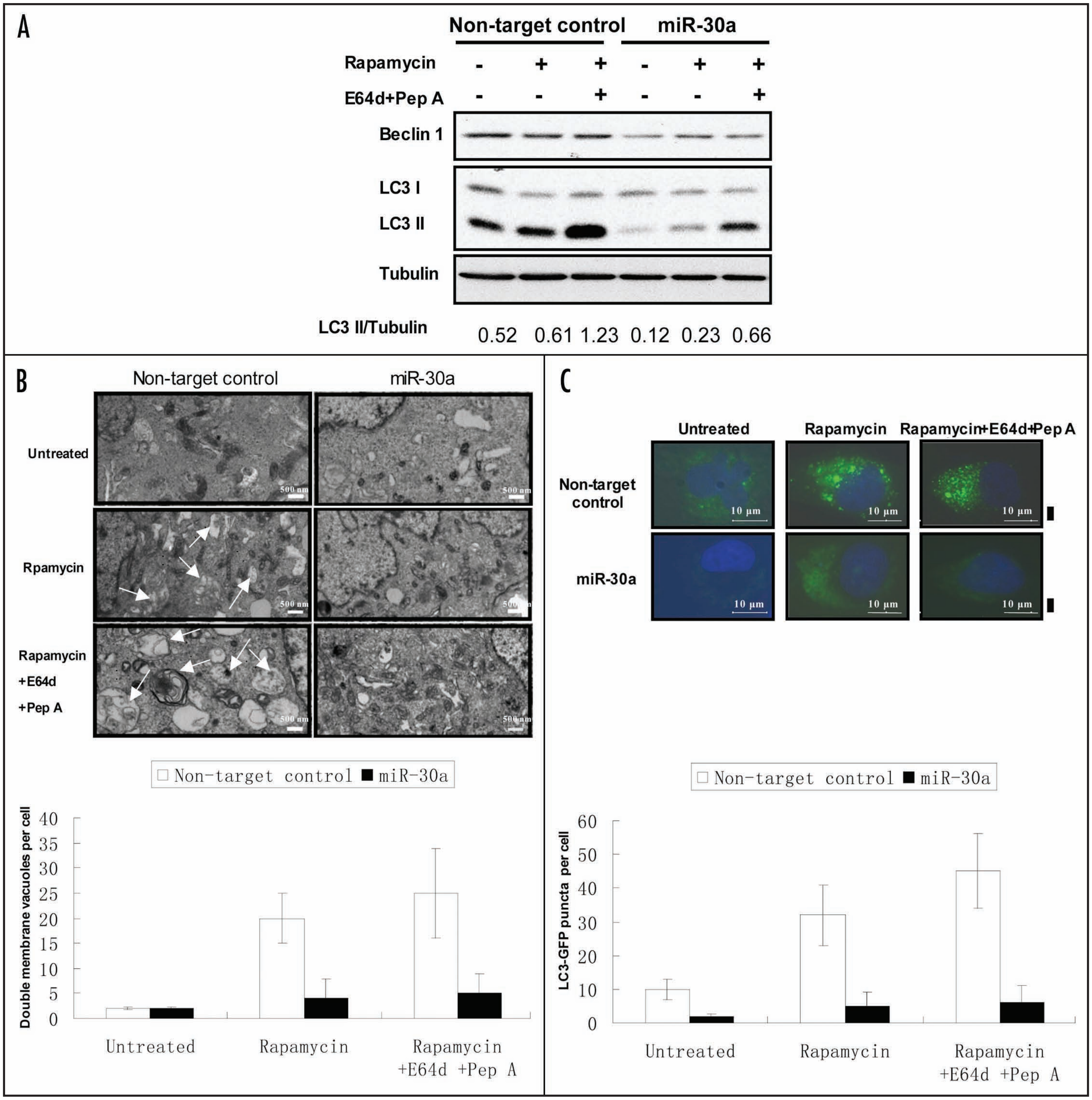

Figure 4. Effect of miR-30a on autophagic response to rapamycin. (A) T98G cells transfected with a mimic of miR-30a or a nontargeting control RNA were treated with rapamycin (200 nM) in the presence or absence of lysosomal protease inhibitors E64D (10 µg/ml) and pepstatin A (10 µg/ml). At the end of treatment, formation of LC3-II was detected by immunoblotting with an anti-MAP-LC3 antibody as described in "Materials and Methods." (B) T98G cells were treated as described in (A), and then were harvested by trypsinization, fixed and embedded in spur resin. Ninety nm-thin sections were cut and examined at 80 Kv with a JEOL 1200EX transmission electron microscope. Arrows indicate autophagic vacuoles. Double membrane vacuoles per cell were determined by counting 20 cells for each sample and average numbers of double membrane vacuoles were shown. The bars are the mean ± S.D. (C) GFP-LC3-expressing T98G cells were treated as described in (A). At the end of treatment, cells were fixed with 4% formaldehyde for 15 min and inspected at 60x magnification for numbers of GFP-LC3 puncta. LC3-GFP puncta per cell were determined by counting 20 cells for each sample and average numbers of puncta per cell were shown. The bars are the mean ± S.D. Results shown are the representative of three identical experiments.

adequate size of samples to make a sound conclusion. However, it remains to be determined whether or not miR-30a also participates in regulating *beclin 1* expression and autophagy in nonmalignant cells. Involvement of miRNA in regulating autophagy is undoubtedly worth further investigation, and these studies should yield new insights into how autophagy is regulated, and the association of this cellular response with various diseases.

The evidence for the importance of both autophagy and miRNAs in cancers has been emerging in recent years. It is becoming increasingly recognized that altered autophagy is associated with tumor formation and progression, and with the altered response to several forms of cancer treatments.[34] For example, we and others observed that autophagy played an essential role in survival of malignant cells under environmental, metabolic or therapeutic stress conditions.[35,36] We found that induction of autophagy protects cancer cell viability under cellular stress such as nutrient deprivation.[35] Katayama et al. reported that autophagy-associated ATP surge protected cancer cell viability and contributed to resistance to cytotoxic drugs such as etoposide and temozolomide.[36] Also, aberrant expressions of miRNAs are known to impact on many patho-physiologic processes, including proliferation, apoptosis and stress response,[37,38] and are implicated in tumor initiation, development and progression. For instance, expression of miR-21 has been reported to promote tumor cell proliferation and growth, and these effects are mediated through upregulation of bcl-2 expression.[39] Our recent comparison of miRNA expression profilings between multidrug resistant and drug sensitive cancer cells revealed a differential expression pattern, and we further found that miR-27a and miR-451 are involved in the modulation of expression of *MDR*1 gene, whose product is the multidrug transporter, P-glycoprotein.[25] The results reported here suggest that miRNAs have the potential of modulating autophagy through regulation of the expression of the key autophagy genes such as *beclin 1*, providing evidence for a new role of miRNAs in a cellular process with the importance that has been recognized increasingly in cancer biology.

Materials and Methods

Cell culture and reagents. Human breast cancer cell lines MDA-MB-468 and MCF-7, lung cancer cell line H1299, and glioma cell line T98G were purchased from American Type Culture Collection. MDA-MB-468 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Cat. No. 31053036); MCF-7 and H1299 cells were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Cat. No. 22400089); and T98G cells were maintained in Ham's F-10 (Invitrogen Life Technologies, Cat. No. 11550043)/DMEM (10:1) medium. All the media contained 10% (v/v) fetal bovine serum (Sigma, Cat. No. F4135), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen Life Technologies, Cat. No. 15140163). Cell lines were cultured in a 5% CO_2-humidified incubator at 37°C.

The mimic (Cat. No. C-30050503) and antagomir (Cat. No. IH-300505-05) of miR-30a and a control miRNA (Cat. No. CN-001000-01) were purchased from Dharmacon Inc., Hank's buffered salt solution (HBSS) was purchased from Invitrogen

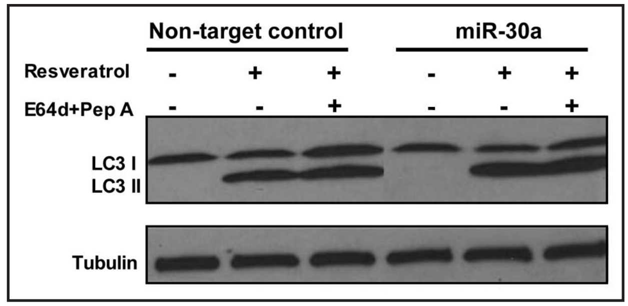

Figure 5. miR-30a does not affect the resveratrol-induced *beclin 1*-independent autophagy. MCF-7 cells transfected with a mimic of miR-30a or a nontargeting control RNA were treated with resveratrol (50 µM) in the presence or absence of lysosomal protease inhibitors E64D (10 µg/ml) and pepstatin A (10 µg/ml). At the end of treatment, formation of LC3-II was detected by immunoblotting with an anti-MAP-LC3 antibody as described in "Materials and Methods."

Life Technologies. (Cat. No. 14025092). Other reagents were purchased from Sigma-Aldrich.

MiRNA microarray profiling. Total RNAs from cells were extracted using TriZol Reagent (Invitrogen Life Technologies, Cat. No. 10296010). RNA labeling and hybridization on miRNA microarray were performed as described previously.[24] Briefly, 5 µg of total RNA from each sample was biotin-labeled by reverse transcription using 5' biotin end-labeled random octomer oligo primer. Hybridization of biotin-labeled cDNA was carried out on miRNA microarray chip (OSU version 4.0, Ohio State University, Columbus, OH), which contains 1,600 miRNA oligo probes derived from 474 human and 373 mouse miRNA genes and printed in duplicates. Hybridization signals were detected by biotin binding of a Streptavidin-Alexa 647 conjugate using an Axon Scanner 4000B. The images were quantified using GenePix 6.0 software (Axon Instrument Inc.,).

Real-time RT-PCR analysis of miRNA. TaqMan® miRNA assays were performed to determine the endogenous mature miRNA expression. Briefly, miRNAs were converted to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4366596), followed by TaqMan® based quantative PCR on the Stratagene 3005P Real-TimePCR system using the miRNA-specific primers from Applied Biosystems. Small nuclear RNA (RNU66) was used as internal control. The expression level of miRNA was calculated using MxPro software (Version 4.00, Stratagene).

Dual luciferase reporter assay. The 1,574 bp fragment of the *beclin 1* 3'-UTR containing the miR-30a targeting sequence (GTTTACA) was cloned into the psiCHECK™-2 dual Luciferase reporter plasmid (Promega, Cat. No. C8021) at the 3' end of the coding sequence of *R. reniformis* luciferase to produce psiCHECKTM2-WT-*BECN*-3'-UTR using PCR (primer Forward: 5'GCA AGC CAG ACA GGA AAA AG3'; Reverse: 5'AAC ATC AAG GGG GAA AAT CC3'). To produce the deletion mutant (psiCHECKTM2-MT-*BECN*-3'-UTR) of the miR30a targeting site, mutagenesis PCR was performed with the following condition: 15 cycles of 30 s at 95°C, 15 cycles of 30 s at 55°C, and 2 min/kb template plasmid DNA at 68°C

(BECN1 miR-30a mutagenesis primer Forward: 5'-CCT TAA CGA AAA TTT CCT ATG TCT GTC TAT TGG TAT GC-3'; BECN1 miR-30a mutagenesis primer Reverse: 5'-GCA TAC CAA TAG ACA GAC ATA GGA AAT TTT CGT TAA GG-3'). The accuracy of the plasmid inserts was determined by complete sequencing analysis.

For the reporter assays, cells were cultured to approximately 80% confluence in a 6-well plate, and then cotransfected with either psiCHECKTM2-WT-*BECN*-3'-UTR or psiCHECKTM2-MT-*BECN*-3'-UTR vector and the miR-30a mimic for 48 or 72 hours. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega, Cat. No. E1910), and Renilla luciferase activity was normalized to firefly luciferase activity.

MiRNAs transfection. Cells in exponential phase of growth were plated in 60 mm plates at 1×10^6 cells/plate and cultured overnight, then transfected with a mimic or antagomir of miR-30a, or a nontargeting control RNA (100 nM) using Lipofectamine 2000 and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Cat. No. 51985034), according to the manufacturer's protocol. The effects of miR-30a and the nontarget control RNA were examined by western blot and real-time RT-PCR.

Western blot analysis. Cells were washed with PBS containing Protease Inhibitor Cocktail (Pierce Biotechnology Inc., Cat. No. 78429), and then lysed in CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, Cat. No. C3228). The lysates were transferred to 1.5 ml Eppendorf tubes and clarified by centrifugation at 16,000 xg for 25 min at 4°C. Equal amounts (25 μg) of cell lysates were resolved by SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were incubated in 5% nonfat milk in TBST-0.1% Tween-20 at room temperature for 1 h, then immunoblotted with the respective antibodies. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Cat. No. 32209). Quantifications of protein bands were performed using the ImageJ software (http://rsb.info.nih.gov/ij).

Real-time RT-PCR. Total RNAs were extracted from cells with TriZol Reagent (Invitrogen Life Technologies, Cat. No. 10296010) following the manufacturer's protocol. First strand cDNA synthesis and amplification were performed using Omniscript RT Kit (QIAGEN, Cat. No. 205113). The following human *beclin 1* primers were used: forward: 5'-CAA GAT CCT GGA CCG TGT CA-3'; reverse: 5'-TGG CAC TTT CTG TGG ACA TCA-3'.[11] The β-*actin* primers were as follows: forward: 5'-GCC AAC ACA GTG CTG TCT GG-3'; reverse 5'-GCT CAG GAG GAG CAA TGA TCT TG-3'.[25] SYBR Green quantitative PCR amplifications were performed on the Stratagene 3005P Real-TimePCR system. Reactions were carried out in a 25-μl volume containing 12.5 μl of 2x SYBR Green PCR Master Mix. The thermal profile for the real-time PCR was 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 59°C for 30 s and 70°C for 30 s. The fold-changes for *beclin 1* expression were calculated using the MxPro software (Version 4.00, Stratagene).

Fluorescence microscopy. Cells were cotransfected with GFP-LC3 vector (1 μg/ml) and a miR-30a mimic (100 nM) using Lipofectamine 2000 (Invitrogen Life Technologies, Cat. No. 11668019). Forty-eight hours later, cells were fixed in 4% formaldehyde for 10 minutes. Cells were then washed thrice with PBS, and observed under a fluorescence microscope (Zeiss Axiovert 200M) with 400x lens.

Electron microscopy. Cells harvested by trypsinization were fixed in 2.5% gluteraldehyde/4% paraformaldehyde in 0.1 M cacodylate buffer, and then post-fixed in 1% osmium tetroxide buffer. After dehydration in a graded series of acetone, the cells were embedded in spur resin. Thin sections (90 nm) were cut on a Reichert Ultracut E microtome. Sectioned grids were stained with saturated solution of uranyl acetate and lead citrate. Sections were examined at 80 kV with a JEOL 1200EX transmission electron microscope.

Note

Supplementary material can be found at:
www.landesbioscience.com/supplement/ZhuAUTO5-6-Sup

Acknowledgements

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Cytoprotective Effect of the Elongation Factor-2 Kinase-Mediated Autophagy in Breast Cancer Cells Subjected to Growth Factor Inhibition

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Abstract

Background: Autophagy is a highly conserved and regulated cellular process employed by living cells to degrade proteins and organelles as a response to metabolic stress. We have previously reported that eukaryotic elongation factor-2 kinase (eEF-2 kinase, also known as Ca^{2+}/calmodulin-dependent protein kinase III) can positively modulate autophagy and negatively regulate protein synthesis. The purpose of the current study was to determine the role of the eEF-2 kinase-regulated autophagy in the response of breast cancer cells to inhibitors of growth factor signaling.

Methodology/Principal Findings: We found that nutrient depletion or growth factor inhibitors activated autophagy in human breast cancer cells, and the increased activity of autophagy was associated with a decrease in cellular ATP and an increase in activities of AMP kinase and eEF-2 kinase. Silencing of eEF-2 kinase relieved the inhibition of protein synthesis, led to a greater reduction of cellular ATP, and blunted autophagic response. We further showed that suppression of eEF-2 kinase-regulated autophagy impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival, and enhanced the efficacy of the growth factor inhibitors such as trastuzumab, gefitinib, and lapatinib.

Conclusion/Significance: The results of this study provide new evidence that activation of eEF-2 kinase-mediated autophagy plays a protective role for cancer cells under metabolic stress conditions, and that targeting autophagic survival may represent a novel approach to enhancing the effectiveness of growth factor inhibitors.

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Introduction

Autophagy is a highly conserved process by which cytoplasm and organelles are digested via autophagosomes and autolysosomes and cellular components are recycled for energy utilization [1,2]. During starvation or growth factor deficiency, autophagy may serve as a temporary survival mechanism by providing an alternative energy source. Autophagy can also optimize nutrient utilization in rapidly growing cells when faced with hypoxic or metabolic stresses, thus contributing to cancer cell survival [3,4,5]. eEF-2 kinase, a Ca^{2+}/calmodulin-dependent protein kinase, acts as a negative regulator of protein synthesis: this kinase phosphorylates eEF-2, a 100 kDa protein that mediates the translocation step in peptide-chain elongation by inducing the transfer of peptidyl-tRNA from the ribosomal A to P site; phosphorylation of eEF-2 at Thr56 by eEF-2 kinase decreases the affinity of the elongation factor for ribosome and terminates elongation [6]. Our previous studies demonstrated that eEF-2 kinase might be a central component of the mammalian macroautophagy pathway that is activated in response to nutrient deprivation [7,8]. The role of eEF-2 kinase in the regulation of stress-induced autophagy has further been confirmed by others [9]. Since protein synthesis is a major energy-consuming process, termination of protein synthesis and induction of autophagy via activation of eEF-2 kinase should conserve energy and support cell survival during time of metabolic stress. Moreover, eEF-2 kinase has been found to be overexpressed and its activity increased in multiple breast cancer cell lines and human breast cancer specimens as compared to adjacent normal tissue [10].

The members of the epidermal growth factor receptor (EGFR) family such as EGFR/HER1 and HER2/erB2 represent attractive targets for therapeutic intervention in treatment of cancer, due to the roles of these receptor tyrosine kinases in stimulating oncogenic signaling pathways and in the development and progression of cancers [11,12,13]. Aberrant expression or activity of the EGFR family receptor tyrosine kinases is encountered in many types of malignancies including breast cancers. Indeed, the EGFR tyrosine kinase inhibitors such as lapatinib and gefitinib, and the HER2/neu-targeted agent trastuzumab, have been shown to possess notable antitumor activity in several types of cancers [14]. These drugs can specifically bind to the receptors with high affinity, resulting in blockade of the downstream signaling pathways and inhibition of tumor growth. Nevertheless, refractoriness to these growth factor inhibitors is common [15,16]. For instance, in patients with HER2-positive metastatic breast cancers, the response rate of trastuzumab is only ~26% [17]. Thus, understanding of the mechanisms underlying the insensitivity to the growth factors inhibitors and developing approaches to sensitizing tumor cells will make these drugs more valuable in treating patients with cancer. In this study, we sought to determine whether activation of eEF-2 kinase-mediated autophagy altered sensitivity of human breast cancer cells to inhibition of growth factor-initiated signaling, and whether modulating autophagy via targeting eEF-2 kinase would render tumor cells more susceptible to the effect of growth factor inhibitors. Our study shows that the eEF-2 kinase-mediated autophagy plays a cytoprotective role in breast cancer cells treated with growth factor inhibitors, and inhibiting autophagic survival can modulate sensitivity to these therapeutic agents.

Results

Nutrient deprivation and growth factor inhibitors activated autophagy in human breast cancer cells

To determine the effect of metabolic stress on autophagy, we first treated the human breast cancer cells MCF-7 with DPBS, and then examined autophagic activity in the treated cells. As shown in Fig. 1A, nutrient deprivation increased the level of LC3-II, a specific marker of autophagic activity. Autophagosome formation was confirmed by GFP-LC3 puncta localization (Fig. 1B). We further found that MCF-7 and MDA-MB-468 breast cancer cells treated with the growth factor inhibitors, gefitinib and lapatinib, showed an increased in LC3-II amount in a dose-dependent manner, as determined by Western blot (Fig. 1C and D). The effects of gefitinib and lapatinib on autophagy were verified using GFP-LC3 cleavage assay (Fig. 1E). These observations suggest that activation of autophagy may represent a cellular response to metabolic stress, including treatment with growth factor inhibitors.

Nutrient deprivation caused a reduction of protein synthesis and cellular ATP

To ascertain whether a causal relationship exists between activation of autophagy and metabolic stress in breast cancer cells, we measured protein synthesis activity and ATP level in MCF-7 cells subjected to nutrient starvation. As shown in Fig. 2, treatment of the cells with DPBS (Dulbecco's Phosphate-Buffered Saline) caused a marked decrease in protein synthesis (Fig. 2A) and ATP content (Fig. 2B). Cellular response to energy stress was also evidenced by an increased phosphorylation on Thr172 of AMP-activated protein kinase (AMPK), an intracellular energy sensor that is activated when cellular energy level decreases (Fig. 2C).

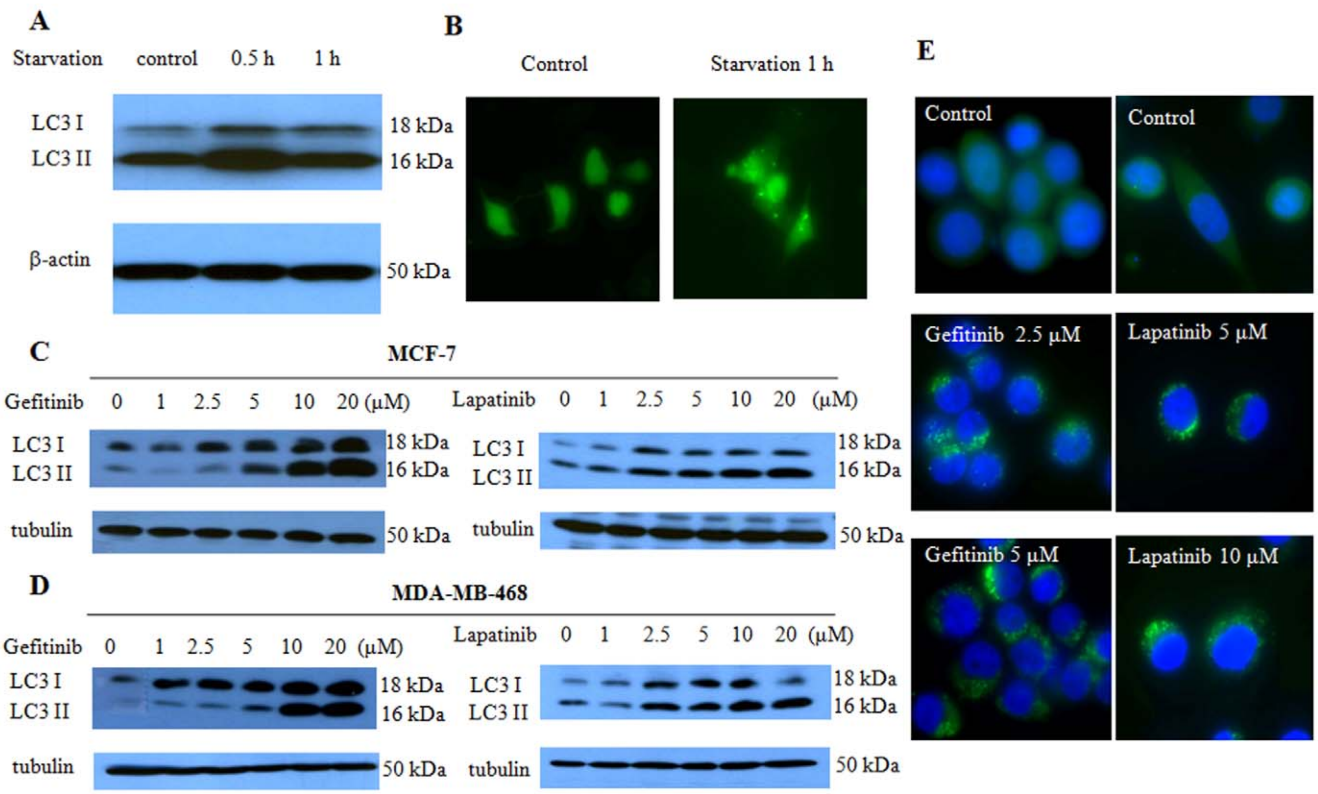

Figure 1. Effect of nutrient deprivation and growth factor inhibitors on autophagy in human breast cancer cells. (**A**) MCF-7 cells were treated with DPBS for the indicated times. At the end of treatment, formation of the autophagy marker LC3-II was detected by immunoblotting with an anti-MAP-LC3 antibody. (**B**) MCF-7 cells transfected with 3 μg of GFP-LC3 plasmid were treated or untreated with DPBS for 1 h, and then observed under a fluorescent microscope. A representation of GFP-LC3 positive cells was shown. (**C, D**) MCF-7 (C) or MDA-MB-468 (D) cells cultured in medium containing 10% FBS were treated with 0.5, 1, 2.5, 5 or 10 μM gefitinib or lapatinib for 24 h, and the LC3-II level was examined by immunoblotting. (**E**) MDA-MB-468 cells were transfected with a GFP-LC3-expressing vector, and then treated with the indicated concentration of gefitinib or lapatinib in the presence of the lysosomal protease inhibitors E64d (10 μg/ml) and pepstain A (10 μg/ml). At the end of treatments, cells were fixed with 4% formaldehyde for 15 min. To determine the autophagic response, cells were inspected at 60× magnification for numbers of GFP-LC3 puncta.
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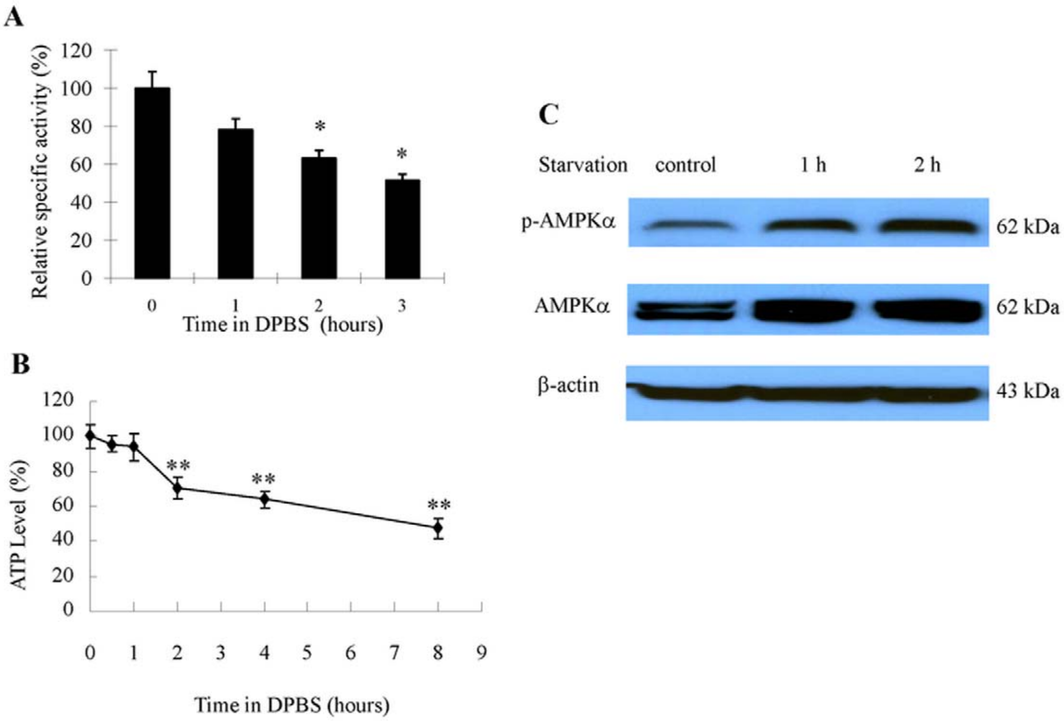

Figure 2. Effect of nutrient deprivation on protein synthesis and cellular ATP in MCF-7 cells. (**A**) MCF-7 cells were treated with DPBS for the indicated times and the rate of protein synthesis was measured by labeling the cells with 25 μCi/ml of EasyTag EXPRESS [^{35}S] protein labeling mix and liquid scintillation counting, as described in "Material and Methods". The specific activity of protein synthesis was determined by the amount of incorporated ^{35}S-methionine/cysteine per mg of total protein per min, and relative activities at indicated times of starvation were calculated as percent of control. Results shown are the mean ± SD of quadruplicate determinations from one of three identical experiments; *$p<0.05$, *t*-test. (**B**) MCF-7 cells were treated with DPBS for the indicated times and ATP content was measured using the ATPlite™ Luminescence Assay Kit. (**C**) AMPK activity was determined by Western blot analysis of phospho-AMPK using an anti-phospho-AMPK antibody, as described in "Material and Methods". Actin was used as a loading control. Results shown are the representative of three similar experiments; each bar or point represents mean ± SD of quadruplicate determinations. Results shown are the mean ± SD of quadruplicate determinations from one of three identical experiments; **$p<0.01$, *t*-test.
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Nutrient deprivation activated eEF-2 kinase through the mTOR/S6 kinase pathway

We next determined the effects of nutrient deprivation on the activity of eEF-2 kinase, a unique calmodulin-dependent enzyme that inhibits protein synthesis and activates autophagy, and on the signaling molecules associated with the regulation of the activity of this kinase. Fig. 3A shows that treatment of MCF-7 cells with DPBS activated eEF-2 kinase, as measured by the phosphorylation of EF-2, the substrate for the kinase. Activation of eEF-2 kinase was also manifested in an increased auto-phosphorylation on Ser398, which is known to positively regulate the activity of this kinase, and in a decreased phosphorylation of the kinase on Ser366, a site known to negatively regulate the activity of this enzyme (Fig. 3A). The activity of S6 kinase, a key translation controller downstream of mTOR, was decreased, as evidenced by a decrease in the phosphorylation on Thr389 of S6 kinase (Fig. 3B). The activity of 4EBP1, a translation repressor, was increased, as shown by a decrease in the phosphorylation of this protein (Fig. 3B).

eEF-2 kinase was involved in autophagy induction and ATP reduction in response to nutrient depletion

To analyze whether eEF-2 kinase plays a regulatory role in nutrient starvation-induced reduction of protein synthesis and ATP content and in activation of autophagy in breast cancer cells stressed with nutrient depletion, we silenced EF-2 kinase expression using siRNA, and then measured ATP level, protein synthesis and autophagy activity following treatment with DPBS. As shown in Fig. 4A, inhibition of eEF-2 kinase by siRNA decreased starvation-induced autophagy. Inhibition of eEF-2 kinase also resulted in mitigation of the nutrient depletion-induced inhibition of protein synthesis (Fig. 4B) and led to further reduction of ATP levels (Fig. 4C). These results further support a role for eEF-2 kinase in activating autophagy in metabolically stressed tumor cells.

Suppression of autophagy decreased growth and survival of metabolically stressed breast cancer cells

To further test our hypothesis that autophagy plays a pro-survival role in response to a compromised supply of cellular nutrients and growth factors during breast cancer development and progression, we knocked down eEF-2 kinase, beclin-1 or ATG5 (two of the key autophagy-related genes) in MCF-7 cells (Fig. 5A), and then compared the growth and survival of these autophagy-deficient cells with that of the cells transfected with a non-targeting RNA in serum-free medium or HBSS. As shown in Fig. 5B, suppression of autophagy by knockdown of those autophagy-regulated genes hindered the tumor cell growth in the absence of serum. Knockdown of eEF-2 kinase, beclin-1 or ATG5 also caused more death of MCF-7 cells cultured in HBSS (Fig. 5C). The autophagy inhibitor, 3-MA, was used as a control and showed a similar inhibitory effect on cell growth and survival in the absence of serum or nutrients (Fig. 5B and C).

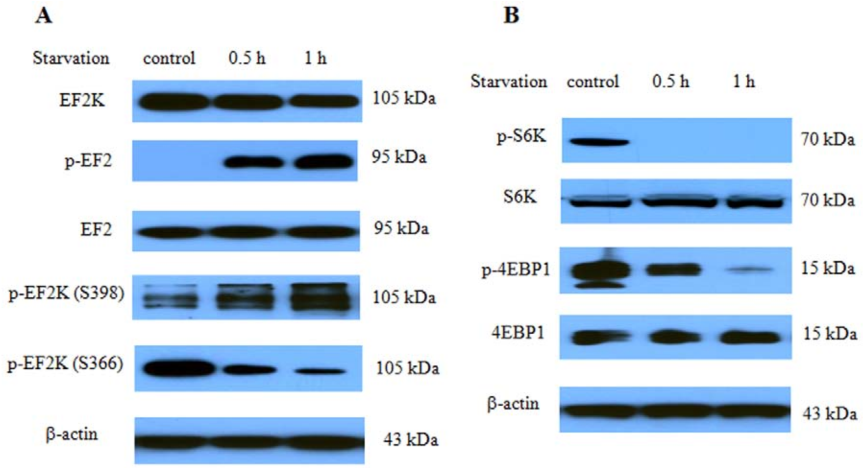

Figure 3. Effect of nutrient deprivation on eEF-2 kinase activity and the associated signaling molecules. (**A**) MCF-7 cells were treated with DPBS for the indicated times, and the levels of eEF-2 kinase, p-EF2, EF2, p-eEF2 kinase (S398) and p-eEF2 kinase (S366) were examined by Western blot using the respective antibodies. (**B**) MCF-7 cells were treated with DPBS for the indicated times, and p-S6 kinase, S6 kinase, p-4EBP1, and 4EBP1 were examined by Western blot using the respective antibodies. β-actin was used as a loading control. Results shown are the representative of three similar experiments.
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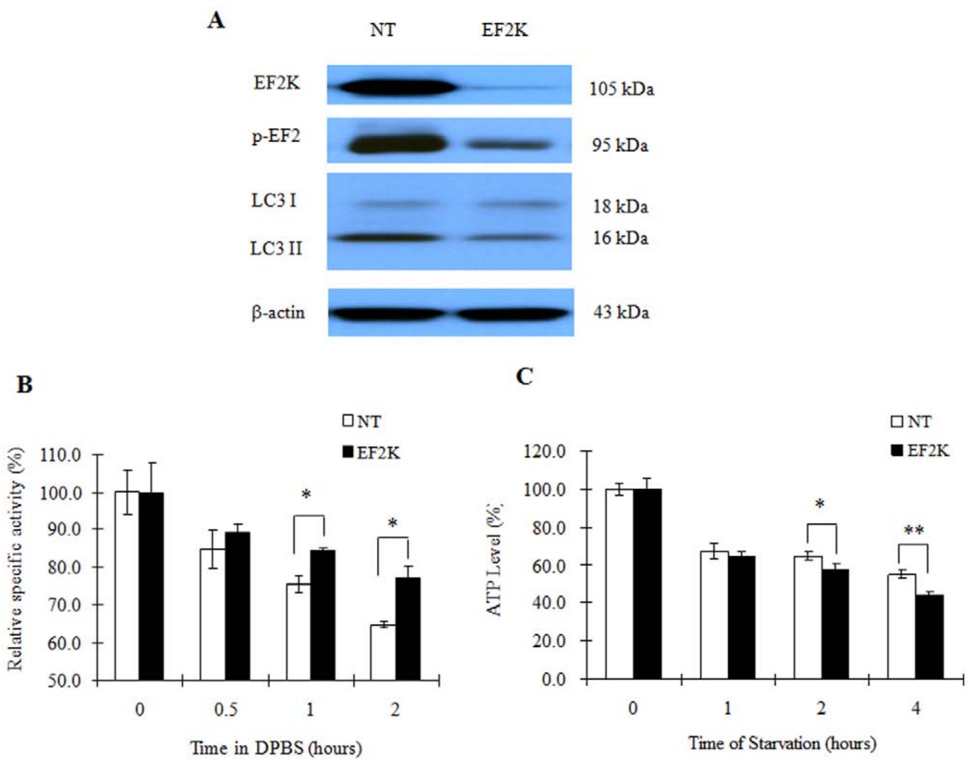

Figure 4. Inhibition of eEF-2 kinase blunts autophagy, mitigates inhibition of protein synthesis and hastens reduction of cellular ATP. (**A**) MCF-7 cells were transfected with a non-targeting RNA or an eEF-2 kinase-targeted siRNA (100 nM) for 72 h, and then treated with DPBS for 1 h. eEF-2 kinase, phosphor-EF-2, and the autophagy marker, LC3-II, were detected by Western blot. (**B**) MCF-7 cells with or without silencing of eEF-2 kinase were treated with DPBS; at the indicated times, cells were harvested for protein synthesis assay. Results shown are the mean ± SD of quadruplicate determinations from one of three identical experiments; $*p<0.05$, *t*-test. (**C**) MCF-7 cells transfected with 50 nM of NT RNA or an eEF-2 kinase siRNA were seeded in 96-well tissue culture plates (1×10^4 cells per well). Forty-eight h later, the cells were starved in DPBS for 1 h, 2 h and 4 h. Cells were collected at the end of starvation for ATP assay. Results shown are the mean ± SD of quadruplicate determinations from one of three identical experiments; $*p<0.05$, $**p<0.01$, *t*-test.
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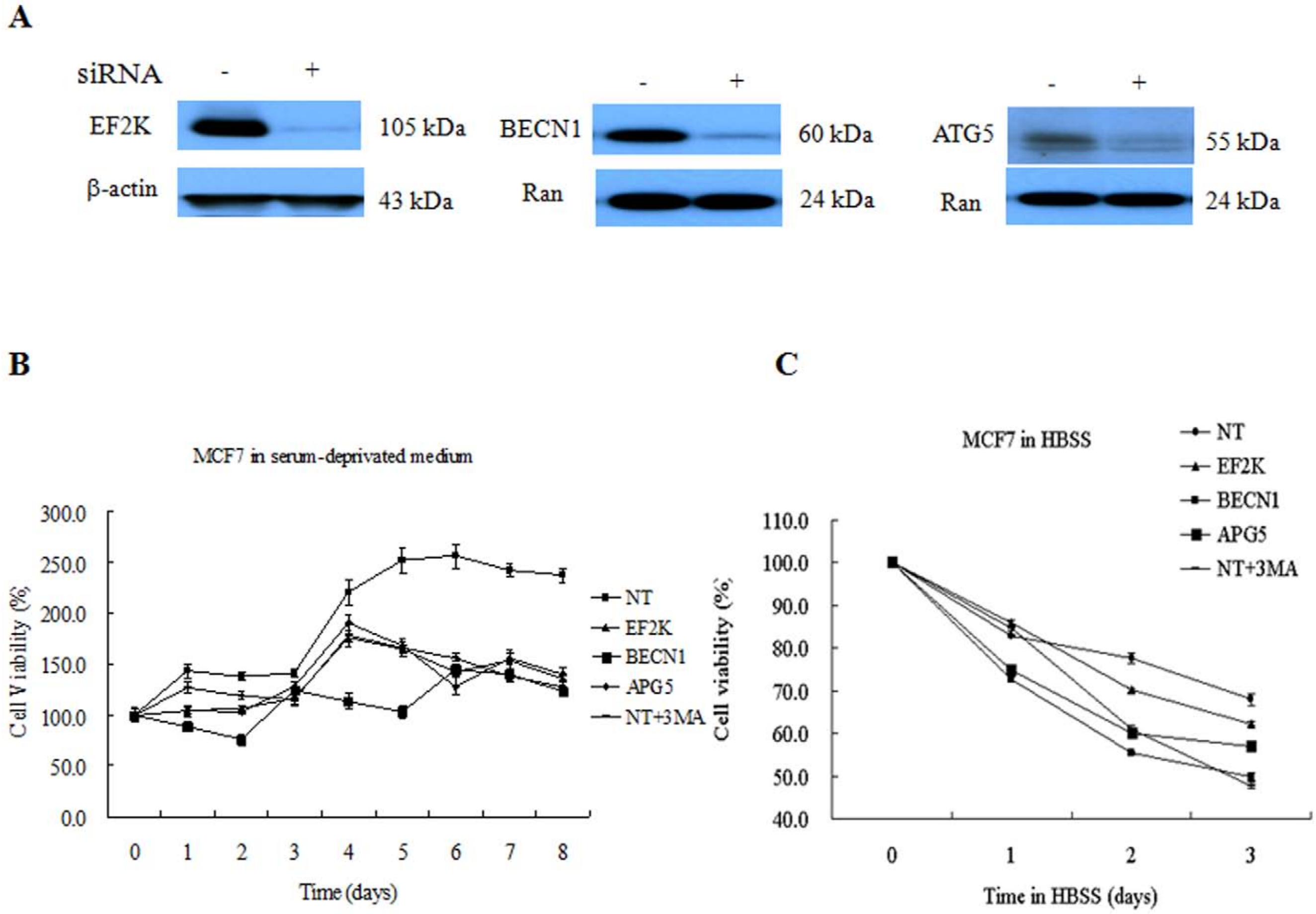

Figure 5. Effects of autophagy suppression on growth and survival of human breast cancer cells. (**A**) MCF-7 cells were transfected with a non-targeting RNA or a siRNA targeting eEF-2 kinase, beclin1, or ATG5. Expressions of eEF-2 kinase, beclin1, or ATG5 were determined by Western blot using the respective antibodies. β-actin or Ran was used as a loading control. (**B**) MCF-7 cells treated with 3-MA or with siRNA targeting eEF-2 kinase, beclin1, ATG5 or a non-targeting RNA were seeded with 10% FBS RPMI 1640 medium in 96-well culture plates (3×10^3 cells per well). After overnight incubation, medium was changed to serum-free medium. Cell viability was determined at the indicated times using MTT assay. Results shown are the representative of three similar experiments; each point represents mean $\pm$ SD of quadruplicate determinations. (**C**) MCF-7 cells treated with 3-MA or with siRNA targeting eEF-2 kinase, beclin1, ATG5 or a non-targeting RNA were seeded with 10% FBS RPMI 1640 medium in 96-well culture plates (3×10^3 cells per well). After overnight incubation, medium was changed to HBSS. Cell viability was determined at the indicated times using MTT assay. Results shown are the representative of three similar experiments; each point represents mean $\pm$ SD of quadruplicate determinations.
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Inhibition of eEF-2 kinase sensitized breast cancer cells to growth factor inhibitors

To determine whether suppression of the eEF-2 kinase-mediated autophagy alters sensitivity of tumor cells to growth factor inhibitors that are in clinical use, we first transfected MCF-7 cells with an eEF-2 kinase-targeted siRNA or a non-targeting RNA, and then treated the transfected cells with a series of concentrations of gefitinib or lapatinib. Fig. 6A and B show that silencing of EF-2 kinase expression increased sensitivity of MCF-7 cells to gefitinib and lapatinib. Similar results were observed with the human breast cancer cells MDA-MB-468 (Fig. 6C and D). Inhibition of autophagy by an eEF-2 kinase-targeted siRNA also enhanced the cytotoxic effects of a small molecule EGFR/ErbB-2 inhibitor (EEi) and trastuzumab, an anti-Her2 therapeutic antibody (Table 1). Combined use of the inhibitors of growth factor and autophagy produced combination indexes (CIs) smaller than 1 (Table 1), indicating a synergism between the actions of those treatments.

Discussion

A number of molecular mechanisms have been revealed to contribute to resistance of cancer to drugs that target growth factor receptor-initiated signaling pathways. These known mechanisms include overexpression or mutation of EGFR [18], co-activation of multiple receptor tyrosine kinases [19,20], mutation or alteration in the downstream effectors of EGFR such as loss of PTEN [21], and compensatory pathways that remain active in stimulating PI-3 kinase [22]. Here, we identified the activation of the eEF-2 kinase-mediated autophagy as a new mechanism responsible for the insensitivity of cancer cells to growth factor inhibition. We found that breast cancer cells utilize autophagy to survive nutrient deficiency and growth factor inhibitors, and eEF-2 kinase also plays an important role in the induction of autophagy triggered by growth factor inhibitors. We demonstrate that inhibitors of growth factors such as gefitinib and lapatinib can induce autophagy in breast cancer cells (Fig. 1), and the nutrient depletion-induced autophagy is associated with activation of eEF-2 kinase via the

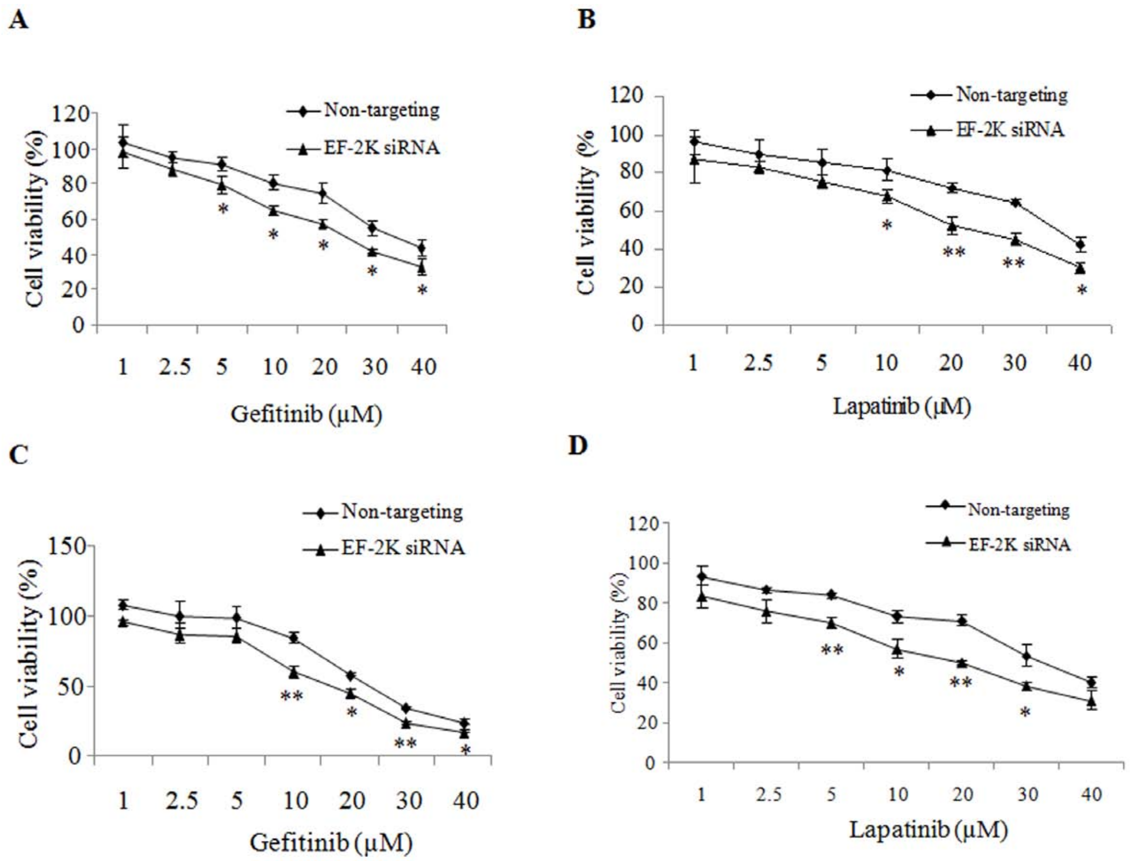

Figure 6. Effects of eEF-2 kinase silencing on sensitivity of human breast cancer cells to growth factor inhibitors. MCF-7 (**A, B**) and MDA-MB-468 (**C, D**) cells transfected with an siRNA targeting eEF-2 kinase or a non-targeting RNA were cultured in RPMI 1640 or DMEM media supplemented with 10% fetal bovine serum at 37°C in a humidified incubator (5% CO_2 and 95% air), and then treated with a series of concentrations of gefitinib (**A, C**) or lapatinib (**B, D**) for 48 h. At the end of treatment, cell viability was determined using MTT assay. Results shown are the representative of three similar experiments; each point represents mean ± SD of quadruplicate determinations; *p<0.05, **p<0.01, *t*-test.
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mTOR/S6 kinase signaling pathways (Fig. 3). Furthermore, we show that inhibiting the eEF-2 kinase-mediated autophagy can sensitize breast cancer cells to growth factor inhibitors (Fig. 6, Table 1). The results of the current study are consistent with our previous findings that eEF-2 kinase plays a critical role in autophagic survival of stressed tumor cells [7,8]. A recent report by Vazquez-Martin et al. shows that autophagy also plays an essential role in the development of resistance to trastuzumab, an anti-Her2 therapeutic antibody, in breast cancer exposed chronically to this agent [23].

Table 1. CompuSyn analysis of the combinations of growth factor inhibitors and eEF-2 kinase siRNA.

Cell types	Samples	CI values
MCF7	Gefitinib: eEF-2K siRNA	0.45
	Lapatinib: eEF-2K siRNA	0.42
	EEi: eEF-2K siRNA	0.51
	Trastuzumab: eEF-2K siRNA	0.60
MDA-MB-468	Gefitinib: eEF-2K siRNA	0.40
	Lapatinib: eEF-2K siRNA	0.53
	EEi: eEF-2K siRNA	0.65
	Trastuzumab: eEF-2K siRNA	0.67

Combination index (CI) was calculated using the computer program, CompuSyn.
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A variety of anticancer therapies induce autophagy, including antiestrogens [24], radiation [25], inhibitors of histone deacetylases [26] and chemotherapy [27,28]. Whether the autophagic response is a pathway to cell death or a mechanism of survival (or both) remains uncertain. For instance, vinblastine increased the number of autophagosomes in pre-neoplastic pancreatic acini [29], whereas pancreatic cancer cells had a decreased autophagic response to vinblastine [30]. Radiation increased autophagy in glioma cell lines, but whether this was a survival or death response remains unclear, since the autophagic changes were independent of sensitivity to radiation [31]. More recently, it was reported that blocking autophagy could enhance the sensitivity of breast cancer to radiation [25]. It is likely that the pro-survival or pro-death role of autophagy is context-dependent.

Several points during breast cancer progression are characterized by a compromised supply of cellular nutrients and growth factors. For instance, when transformed cells invade the stroma or land in a metastatic site, these cells are temporarily "cut off" from a robust blood supply, decreasing access to oxygen, estrogen, glucose, amino acids, and other growth factors. To survive this nutrient-depleted state, breast cancer cells (much like neonates) could utilize autophagy to generate ATP from recycled organelles and long-lived proteins. To avert cell death, autophagic cells must limit protein synthesis to prevent the critical depletion of ATP. This is accomplished through inhibition of eIF4 and activation of eEF2-kinase to inhibit protein synthesis. Indeed, our studies demonstrated that failure to activate eEF-2 kinase blocked autophagy (Fig. 4), handicapped cell growth (Fig. 5B) and hastened cell death (Fig. 5C). Additionally, as recently shown by us [7] and others [32,33], the activity of mTOR and S6 kinase are decreased following nutrient/growth factor deprivation and this relieves the inhibition of eEF-2 kinase [34]. Since protein synthesis devours ATP, termination of protein elongation via activation of eEF-2 kinase should conserve energy and support cell survival during times of starvation. Furthermore, cells depleted of eEF-2 kinase were unable to tolerate nutrient withdrawal as manifested by accelerated loss of viability when grown in serum-free media (Fig. 5), likely due to a critical depletion of ATP because of continued protein synthesis in the face of inadequate nutrients. This may also explain the increased sensitivity to growth factor inhibitors in tumor cells with silencing of eEF-2 kinase (Fig. 6, Table 1). MCF-7 cells are known to be deficient in caspase-3 and haplo-insufficient in *beclin 1* [35,36]; however, in our study we found that the responses of MCF-7 cells to nutrient deprivation or growth factor inhibitors are not different from other breast cancer cell lines such as MDA-MB-468, suggesting that the effects of inhibiting eEF-2 kinase on autophagic response and cell survival/death are independent of caspase-3 and beclin 1.

In summary, the results reported here provide new evidence that activation of eEF-2 kinase and autophagy plays a protective role for breast cancer cells under metabolic stress including growth factor inhibition. By inhibiting autophagy, breast cancer cells should be less able to cope with nutrient/growth factor deprivation, resulting in better therapeutic outcomes for breast cancer patients. Thus, targeting the eEF-2 kinase-regulated autophagic survival may be exploited as a novel approach to preventing and overcoming refractoriness of cancer cells to growth factor inhibitors.

Materials and Methods

Reagents, antibodies and cell lines

Dulbecco's phosphate buffered saline (DPBS) and Hank's balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). E64, pepstatin A, protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), 3-methyladenine (3-MA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trichloroacetic acid, anti-β-actin and anti-ran antibodies were purchased from Sigma-Aldrich (Saint Louis, MO). Anti-eEF-2 kinase, anti-phospho-eEF-2 kinase (S366), anti-eEF-2, and anti-phospho-eEF-2 (T56) antibodies were purchased from Cell Signaling Technologies (Beveley, MA). Anti-LC3B antibody was purchased from Novus Biologicals (Littleton, CO) and anti-BECN1 and anti-ATG5 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-eEF-2 kinase (S398) antibody was a gift from Dr. Chistopher Proud (University of Dundee, UK). Human Breast cancer cell lines MCF-7 and MDA-MB-468 were obtained from American Type Culture Collection (ATCC, Manassa, VA). Cells were maintained in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator (5% CO_2 and 95% air).

siRNA and plasmid DNA transfection

For siRNA transfection, ON-TARGET plus siRNA oligos (Dharmacon, Lafayette, CO) and Oligofectamine (Invitrogen) were diluted with OPTI-MEM I reduced serum medium first, and then incubated for 20 minutes before adding to cell culture. Cells were transfected with siRNA for 24~72 hours. The eEF-2 kinase siRNA (AAGCUCGAACCAGAAUGUCAA) was customer-designed and synthesized by Dharmacon. siRNA targeting human BECN1 (J-0105552-05) or human ATG5 (J-004374-07) and the non-targeting control siRNA (siCONTROL, D-001810-01) were purchased from Dharmacon. For plasmid DNA transfection, cells were tansfected with the mixture of plasmid pEGFP-LC3 and Lipofectamine 2000 (Invitrogen) in OPTI-MEM I reduced serum medium. After 6-hour incubation at 37°C, transfection medium was replaced with RPMI 1640 medium without antibiotics. GFP expression was detected under an inverted fluorescent microscope. Transfectants were selected with 600 μg/mL of G418 (Invitrogen) and maintained with 300 μg/ml of G418.

Western blot analysis

Cells were washed twice with ice-cold PBS and scraped off the tissue culture dishes. Cells were collected in microcentrifuge tubes and centrifuged at 1000×g for 5 minutes. Cell pellets were lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerolphosphate, 1 mM Na_3VO_4, 1x protease inhibitor cocktail, and 1 mM PMSF] and sonicated for 5 seconds. The lysates were clarified by centrifugation at 13,000×*g* for 15 minutes at 4°C. Thirty μg of proteins were resolved by SDS-PAGE and transferred to ImmunoBlot PVDF membrane (Bio-Rad Laboratories, Hercules, CA), and the membranes were probed with the respective antibodies. Pierce ECL Western Blotting Substrate kit (Pierce, Rockford, IL) was used to detect protein signals on the immuno-blots.

Assay of protein synthesis

Protein synthesis rate was measured with a radio-labeling assay [37]. Briefly, cells seeded in 60-mm culture dishes were labeled with 25 μCi per ml of EasyTag EXPRESS ^{35}S protein labeling mix (PerkinElmer, Boston, MA) in RPMI 1640 medium. After incubation at 37°C for 15 min, cells were washed 4 times with 4 ml of ice-cold PBS and then lysed in 200 μl of Complete Lysis-M lysis reagent with 1 Mini Protease Inhibitor Cocktail tablet per 10 ml of lysis reagent (Roche Diagnostics, Indianapolis, IN). Cell lysates were collected in a microfuge tube and clarified by centrifugation at 13000×g for 10 min at 4°C. Supernatant was precipitated with 20% of trichloroacetic acid and collected on GF/C filters (Millipore, Bedford, MA). Filters were washed 4 times with 1 ml of 10% trichloroacetic acid and subject to liquid scintillation counting. The specific activity of protein synthesis was determined by the amount of incorporated ^{35}S-methionine/cysteine per mg of total protein per min.

GFP-LC3 cleavage

Cells were transfected with a GFP-LC3-expressing plasmid. GFP-LC3-expressing cells were subjected to nutrient depletion in the presence of the lysosomal protease inhibitors (50 nM bafilomycin A1, 5 μg/ml E64D, 5 μg/ml leupeptin and 5 μg/ml pepstatin A). At the end of treatment, Cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 15 min. To determine the autophagic response, cells are inspected at 60× magnification for total number of GFP-LC3 puncta. At least 150 cells are scored in each treatment [38].

Measurement of cellular ATP

Cellular ATP was measured using a luminescence ATP detection assay system (ATPlite kit, PerkinElmer, Boston, MA).

The assay was carried out in 96-well cell culture plates according to the manufacturer's instruction. The luminescence was detected on a PerkinElmer Victor 3 plate reader as counts per second (CPS). The relative ATP level was calculated by dividing the CPS of the treated samples by that of control samples.

Cell viability assay

Cell viability was determined using MTT assay. Briefly, cells were seeded in 96-well tissue culture plates and subjected to different treatments. At the end of treatments, cells were incubated with 20 µl of 5 mg/ml MTT reagent. After 4-hour incubation at 37°C in a humidified atmosphere containing 5% CO_2, formazan crystals were dissolved in 200 µl of DMSO. Absorbance at 570 nm was determined using a PerkinElmer Victor3 plate reader.

Author Contributions

Conceived and designed the experiments: YC HL WNH JMY. Performed the experiments: YC HL XR TN. Analyzed the data: YC HL JMY. Wrote the paper: YC HL JMY.

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Abstract – AACR 2007

Targeting Autophagic Survival by Inhibiting Elongation Factor-2 Kinase in Human Breast Cancer Cells

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Autophagy is an evolutionally conserved process employed by cells to degrade proteins and organelles in response to metabolic stress. Cells can recycle amino acids, fatty acids and nucleotides for macromolecular biosynthesis and ATP generation, and by sequestering damaged organelles can prevent the release or accumulation of toxic substances. Autophagy is commonly observed in cancer cells of various origins in response to nutrient deprivation. We described elongation factor-2 kinase (eEF-2 kinase; a.k.a calmodulin-dependent protein kinase III) as a structurally and functionally unique enzyme (Ryazanov et al, *PNAS*, 1998) that is activated by starvation and phosphorylates and inactivates eEF-2, thereby terminating peptide chain elongation. Since protein synthesis is a major energy-consuming process, decreasing protein elongation by activating eEF-2 kinase could be an energy-saving survival strategy. Our laboratory reported that the activity of eEF-2 kinase was increased in many malignant cell lines and certain cancer tissues (Bagaglia et al., *Cancer Res*., 1992), and that knockdown of eEF-2 kinase blocked autophagy and accelerated cell death in human glioblastoma cells (Wu et al, *Cancer Res*, 2005). We now test the hypothesis that eEF-2 kinase plays a critical role in the ability of cancer cells to survive oxygen and nutrient deprivation. MCF-7 human breast cancer cells were transfected with a GFP-tagged LC3 expression vector to track the formation of autophagosomes. Autophagy was induced by nutrient/growth factor deprivation as manifested by autophagosome formation in GFP-LC3- transfected MCF-7 cells. Treatment with a potent and specific inhibitor of eEF-2 kinase, NH125 (Arora et al, *Cancer Res*, 2003), inhibited autophagy as indicated by a reduction in autophagosome formation. In either transient or stable MCF-7 transfectants, NH125 was 10-times more potent and more effective than 3-methyladenine, a known autophagy inhibitor. To determine the effects of blocking autophagy via inhibition of eEF-2 kinase on cellular energetics, we studied the rate and amount of ATP depletion in NH125- and vehicle-treated MCF-7 transfectants. Following nutrient deprivation, inhibition of eEF-2 kinase by NH125 resulted in a greater and more rapid reduction of cellular ATP as compared to vehicle treatment. These results provide additional evidence that eEF-2 kinase is activated in response to metabolic stress, and that inhibiting eEF-2 kinase may overcome the cellular attempts to survive via autophagic regeneration of ATP.

Targeting autophagic survival pathway sensitizes human breast cancer cells to growth factor antagonists

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Malignant cancer cells often face metabolic stresses such as nutrient, growth factor or oxygen scarcity. We previously reported that cancer cells utilized autophagy, a highly regulated cellular degradation process, as a survival strategy to cope with metabolic stress. Elongation factor-2 kinase (eEF-2 kinase), a protein synthesis regulator, phosphorylates and inactivates eEF-2, thereby terminating peptide chain elongation and inhibiting protein synthesis. Since protein synthesis is a major energy-consuming process, down-regulation of this process by activating eEF-2 kinase may be used by cancer cells as an energy-saving survival mechanism. In the current study, we tested the hypothesis that eEF-2 kinase plays a critical role in the ability of breast cancer cells to survive growth factor/nutrient deprivation. Autophagy activity was determined by measuring the formation of LC3 II, and eEF-2 kinase activity by measuring phosphor-eEF-2 using immunoblot analyses. We found that eEF-2 kinase and autophagy were activated following starvation treatment in human breast cancer cell line MCF7. Nutrient starvation also decreased mTOR activity as evidenced by the decreases of phosphor-S6 kinase and phosphor-4EBP1, and reduced the incorporation rate of ^{35}S-methionine, indicating protein synthesis was inhibited. Silencing of eEF-2 kinase by RNA interference (RNAi) relieved the inhibition of protein synthesis, and resulted in a greater reduction of cellular ATP. eEF-2 kinase-targeted RNAi also blunted autophagic response of the tumor cells. Inhibition of autophagy by knockdown of eEF-2 kinase or autophagy-related gene Beclin-1 impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival. These results indicate that in response to nutrient/growth factor deprivation breast cancer cells activates eEF-2 kinase and autophagy to decrease protein synthesis and regenerate ATP, and that inhibition of eEF-2 kinase renders cells continue to elongate peptide, deplete ATP, and impairs cancer cell survival under metabolic stress. Furthermore, we determined whether inhibition of autophagy sensitized breast cancer cells to growth factor antagonists. Synergistic effect on cell growth inhibition was observed from combination of a small molecule EGFR/ErbB-2 inhibitor with an autophagy inhibitor 3-methyladenine in SKBR3 and MDAMB468 cells (combination index values at ED50 0.6279 and 0.7879, respectively). Inhibition of autophagy by knockdown of eEF-2 kinase or Beclin 1 sensitized SKBR3 and MDAMB468 cells to the EGFR/ErbB-2 inhibitor and the mTOR inhibitor rapamycin. These results provide new evidence that activation of eEF-2 kinase and autophagy plays protective role for cancer cells under metabolic stress, and that targeting autophagic survival may represent a novel approach to sensitizing cancer cells to growth factor antagonists.

Regulation of Autophagy by the *Beclin 1*-targeted MicroRNA in Cancer Cells

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Beclin 1, the mammalian homologue of the yeast *Atg6*, is a key autophagy gene whose product is part of class III Phosphoinositide 3-kinase complex that participates in the formation of autophagosome. It is now widely acknowledged that beclin1- mediated autophagy plays an essential role in the regulation of cell survival and death, and implicates in many physiologic and pathophysiologic processes such as aging, cancers, and neurodegenerative diseases. Nevertheless, expression of *beclin 1*, both protein and mRNA, was found to be aberrant in several types of cancers. The mechanism underlying the altered expression of *beclin 1*, however, is largely unknown. The objective of this study was to explore the role of microRNAs (miRNAs) in the regulation of *beclin 1* expression and autophagy. MiRNAs are a class of endogenous, 22 - 24 nucleotides non-coding RNA molecules able to affect stability and/or translation of mRNA, thereby modulating the expression of genes involved in many biological processes. In the current study, we found that miRNA miR-30a could negatively regulate *beclin 1* expression resulting in decreased autophagic activity. We observed that following nutrient depletion or rapamycin treatment, while autophagy was increased, endogenous miR-30a expression was down-regulated in T98G glioma and MDA-MB-468 breast cancer cells, as analyzed by the miRNA microarray expression profiling and qRT-PCR. An in silico search for miRNA binding sites using the PicTar algorithm (http://pictar.bio.nyu.edu) revealed that the 3' - UTR of *beclin 1* contains consensus sequences for miR-30a. To confirm that the miR-30a binding sequences in the 3' - UTR of *Beclin 1* contribute to the modulation of beclin 1 expression, we co - transfected T98G, MDA-MB-468 and H1299 cells with a reporter gene vector (psiCHECK2) containing the wild-type or mutated binding sequences and a mimic of miR-30a, then measured the luciferase activity. We showed that the miR-30a mimic significantly decreased the luciferase activity in cells transfected with the wild-type reporter plasmid, but had no effect on the luciferase activity in cells transfected with the reporter plasmid containing the mutated miR-30a binding sequences. Treatment of cells with the miR-30a mimic also significantly decreased the *beclin* 1 mRNA and protein, as measured by qRT-PCR and Western blot, respectively. Furthermore, inhibition of *beclin 1* expression by the miR-30a mimic blunted activation of autophagy induced by rapamycin, as evidenced by the decreases in LC3-II turnover, formation of GFP-LC3 aggregation and double membrane vacuoles in the cytoplasm. These results demonstrate that *beclin 1* is a target for miR-30a, and that miRNA can control autophagy though modulating the expression of *beclin 1*. Our study of the role of miR-30a in regulating autophagy reveals a novel function for miRNA in a cellular event with significant impacts in cancer development and progression.